

The Cold War's unwanted monument

The US project to build a space station is once again in trouble. The best solution is none of the alternatives presented last week to President Clinton, but a mechanism for true international participation.

THE US space station Freedom has come a long way in the past nine years — at least on paper. The latest development, beginning last week, is that the Clinton administration is attempting to make a choice between three new and alternative designs for the station, each capable of substantially cutting the cost of the project from the recently fashionable estimate of \$30 billion. A few weeks from now, there will be a formal proposal to the Congress, which will then be expected to include an appropriate allowance in the budgets for the next and succeeding financial years (beginning on 1 October). It is already plain that there will be a row. Many in the Congress are fond of the original design, if not for itself then for the jobs it will create in their constituencies. But they and others are also conscious of the need to cut public spending and, with it, the federal fiscal deficit. It matters little what fudge resolves that conflict. It would be better if the Congress took this critical opportunity to ask again what the project is for, and whether it is worth even the \$9 billion or so that the administration seems prepared to spend.

The purpose of the space station has been ambiguous from its conception, when the Cold War still raged. Part of the Reagan administration's case for Freedom was to reassert US leadership in space exploration against the seemingly shaming drumbeat of the endurance records being set by then-Soviet cosmonauts' visits to Mir space stations. Freedom also offered a continuing use for the space shuttle (which, in two of the alternative designs now on offer, will literally be the housing for visitors to the space station for periods up to three weeks). Freedom would also keep men in space. And there was also, of course, to be a scientific programme, the distinctive parts of which — those that cannot be accomplished by other and cheaper means — boil down to the exploitation of the almost zero gravitational field in laboratories in free fall around the Earth. One hope, for example, is that protein molecules may crystallize more readily in such circumstances.

This package of disconnected promises does not justify the spending of \$30 billion, or even a tenth of that. Now that the Clinton administration has said the unsayable by declaring that it considers the space-station project to be worth only about \$9 billion (less than the estimated costs of each of last week's alternatives), it also opened the question of whether the same objectives could be attained more modestly yet more effectively by other means. The shuttle programme, for example, will continue; why not spend a little money to enhance the capabilities of that craft, thus ensuring that there

continue to be men and women in space? And why not divert a little of the money to more effective study of the Earth's climate than will be provided by the satellites the National Aeronautics and Space Administration (NASA) will have in orbit by the end of the decade?

There is, unfortunately, one obstacle in following that path, logical though it may be. NASA has enlisted as partners in its Freedom venture its counterparts in Canada, Europe and Japan, each of which has spent time and money on the design and engineering development of accessories for the shuttle. Even redesign will be annoying and costly for these partners, cancellation would make them seem fools. The administration's price-tag of \$9 billion may, for all we know, be the price it considers worth paying to avoid the ructions cancellation would bring, but even that calculation would be a fudge. Freedom is not strictly the international project it is often represented as, but an *ad hoc* collaboration in which no partner is irrevocably bound. The same difficulty afflicts the Superconducting Super Collider (SSC), to which non-US partners have also been invited without the promise of a voice in design and overall management — and in the knowledge that the US Congress ultimately decides whether or not it survives.

Even now, it is not too late for the US administration to resolve the conflict between the weakness of the case for Freedom and the awkward overseas obligations. After all, none of the governments concerned can be any more eager than the US administration to embark on the expensive phases of the Freedom project that lie ahead. So why not do for the exploration of space by people what is already long overdue in experimental high-energy physics — create an international planning mechanism to consider what might be done, with the understanding that particular projects would be managed as common enterprises regulated by formal treaties? That would be a better marker for the beginning of the next millennium than the purposeless monument to the Cold War now in prospect. □

Universities' own feet

Safeguards are needed if students are to pay a greater share of the costs of their education.

WHEN an official of the Harvard Alumni Association boasted last week, at the university's annual commencement cer-



emony, that more than \$200 million had already been raised this academic year from gifts and from among the 250,000 ex-students scattered around the world, those from other universities were torn between envy and admiration. The Peter principle ("To him that hath shall be given...") seemed to be at work again. Although Harvard, even among private universities, has been cleverly imaginative in looking after its money, it clearly benefits from the intellectual quality of its students and from their earning power. Not every institution of higher education can hope to mimic Harvard's success.

Yet many universities will be forced to try in the years ahead. Everywhere, even where universities are notionally the responsibility of central or regional governments, the current recession has made more evident the gap between public subsidy and educational need. And the inadequacy of resources can, only, other things being equal, get worse. Where universities are supported by governments, spending on higher education must compete with other forms of welfare spending, themselves destined to increase for demographic and other reasons. In Britain, where tuition is free to undergraduates from Britain and other countries within the European Communities, the London School of Economics (LSE) is now proposing to ask future students to make a contribution towards the cost of their education. Other British universities profess disapproval, on the grounds that it would be better to shame the government into being more generous. But they will be keenly interested to see whether the LSE gets away with its small step towards voluntary privatization.

The dangers are not quite what they seem. Universities strong enough to break with a tradition that tuition is free will, almost by definition, be sensible enough to know that they must preserve the quality of their student intake by means of scholarships, and that the financial benefits that fee-payers bring should not undermine academic standards. The real risk is that governments, impatient as always, will seek to push the successful institutions too quickly along the road to partial independence, and that they will impel others in the same direction when the chances of them making the transition successfully can only be minimal. When at last it seems everywhere to have been recognized that higher education for as many as possible is a public good, that would be folly.

A further problem, nowhere satisfactorily solved, is how students in higher education should be helped to afford the costs they will increasingly be asked to bear. Part of the trouble is that students are often legally adult, but usually innocent of employment. In some places (the United States and Britain, for example), there are schemes for making loans to students. Elsewhere (Australia, for example), graduates pay extra tax. None of these schemes is thoroughly equitable, and there is at least a suspicion that all of them discourage some able students from seeking higher education. But the knowledge that the financing of higher education is a problem common to all reasonably prosperous countries should be at least a comfort; the best hope is that it will lead to common solutions, which of necessity cannot be Harvard's way for all.

Half-tax on US energy

President Clinton is heading for a fudge on his planned tax on fuels, likely to finish as a tax on gasoline only.

PRESIDENT Bill Clinton's plan for a better balanced US budget through a blend of higher taxes and reduced spending will be made or broken by the Congress over the next few weeks, but one component of it has already bitten the dust — a general tax on fuel consumption. This tax, the chief source of extra revenue, would have been directly related to the energy content of fossil fuels, for which reason it became known as the 'Btu tax' (after the "British thermal unit" still uniquely used as a unit of energy in the United States).

In the few weeks after the appearance of the budget, Washington's army of special-interest lobbyists had canvassed a host of reasons why the tax would be unfair to their constituents, the poor and the elderly, for example, not to mention international shippers and airlines whose competitiveness would allegedly be impaired. By last week, the president was letting it be known that he cared little how the tax was levied provided that it produced some revenue. The outcome may be an extra federal tax on gasoline, to which there will no doubt be attached a variety of exceptions. That is a cop-out.

On this occasion, there is no reason to suppose that the aversion to the general energy tax has been provoked by the name of the energy unit and by the traditional attitude of the United States towards taxes with British connotations (but there is a case for the United States measuring energy in the units that other people use). Rather, the objections have stemmed from a deep suspicion of taxes that are generally applicable. But Clinton's original purpose was not simply to raise revenue but also to make a start on reducing US emissions of greenhouse gases, for which purpose only a generally applicable tax can mould the pattern of fuel consumption in economical directions. To behave otherwise is to pretend that the effects of molecules of greenhouse gases depend on the purposes for which they were formed as combustion products. But sadly, carbon dioxide molecules from an old people's home are indistinguishable from those from gas-guzzling motor cars.

Ideally, of course, a greenhouse tax should be linked with carbon content. That would imply that natural gas would be less heavily taxed than coal, joule for joule, and that electricity generated by wind-power would not be taxed at all. (In the next few weeks, the farming lobby in Washington can be counted on to fight for exemptions for ethanol produced from sugar, pleading the threat of global warming.) It would have been instructive for us all if the US Congress had been given the task of examining the greenhouse relationships between different fuels; that is the kind of task its committees do well. Indeed, there is a case for starting right away. However much or little revenue the administration squeezes out of the Congress in the next few weeks, the president will be back with a greenhouse tax before very long.

Fate of space station hangs on nominations of US Congress

Washington. A final decision on the future of the space station Freedom is fated to drop into the unsteady hand of the US Congress after a three-month redesign process demanded by President Clinton ended inconclusively.

Clinton was expected to identify one option for further investigation earlier this week. When the White House instigated the redesign process just over three months ago, officials let it be known that failure

of taxpayers' money. Option C, the "man in the can", is a fresh non-modular design — although its supporters claim that much of the \$9 billion so far spent on Freedom has been invested in systems engineering work that would still be relevant if C were chosen.

The space station lobby and its adherents in Congress, led by George Brown (Democrat, California), chair of the House of Representatives' Science, Space and Technology Committee, have made clear their strong support for continuity, as expressed in Option B. Some congressmen held last week that B is the only option that could gather a sufficiently strong coalition of support in Congress to survive.

Expert opinion seems to contradict that view. A special panel under the chairmanship of Dr Charles M. Vest, president of the

Massachusetts Institute of Technology, met last week to advise the president on the outcome of the redesign. Vest concluded that B "carried unnecessary system complexity" and said that the choice should be between A and C.

But NASA administrator Dan Goldin, who was brought in from private industry to shake up the much-maligned administration, is said strongly to favour C. At a congressional hearing last week, he was asked by Sam Johnson (Republican, Texas) why, if he was neutral as he claimed, the slides for Option C were in colour and the others in black and white. Goldin said he had not prepared the graphics himself.

In a stormy session before the science subcommittee, Goldin was repeatedly pressed by James Sensenbrenner (Republican, Wisconsin) on an allegation that he had ordered the destruction of charts showing the consequences of the three options on jobs in the aerospace industry. Goldin said — four times — that he "did not recall" giving such an order.

Whichever option is chosen, the president's advisory panel says that the orbital inclination of the space station should be raised from 28.8 degrees to 51.6 degrees, which will make it accessible to Russian launch sites, but will reduce the payload which the US space shuttle can carry to the station.

The space lobby is banking on heavier Russian participation in the programme to reduce costs. One possibility would be to merge the modular Option A with the Russian's proposed Mir 2 station, due for launch in 1997. A less likely choice would be to involve Russians in the construction and launch the 'big can' of Option C, which might save money but would further undermine congressional support.

Supporters of the programme are now very much on the defensive. Some sympathetic congressmen are even falling back on familiar warnings that withdrawal from the project would leave the Russians in the driving seat of manned space exploration.

The future of the space station will now rest on a series of debates in Congress, beginning this week with a debate on the floor of the House of Representatives, when opponents of the project plan to move to kill it.

If Clinton publicly withdraws his support for the space station, the project will almost certainly die. But if he continues to offer muted support for it, the question may drift on until August, and will be decided by the key budget votes that must at that point be taken.

Colin Macilwain

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Option A for Space Station Freedom redesign (above); Daniel S. Goldin, NASA administrator (right).

to meet a budgetary ceiling of \$9 billion for a permanently manned facility would result in withdrawal of support for the programme.

On its completion last week, the redesign process yielded three options ranging in cost from \$11.9 to \$13.3 billion. Uncertainty now arises because the numbers are not so far above the ceiling set by the president that he will be compelled to make a clear-cut decision to end the programme, in the process offending important constituencies in California and Texas and leaving blood on his hands.

The redesign, conducted at break-neck speed by engineers of the National Aeronautics and Space Administration (NASA), examined the feasibility of three options. Option A is a version of the Space Station Freedom with a much simpler and less ambitious control system, and possibly supported by an existing, military satellite called Bus-1. Option B is a shrunk version of Freedom that retains its basic design and therefore uses the design elements on which NASA has already expended \$9 bil-

lion of taxpayers' money. Option C, the "man in the can", is a fresh non-modular design — although its supporters claim that much of the \$9 billion so far spent on Freedom has been invested in systems engineering work that would still be relevant if C were chosen.

This development left space station supporters rallying to the less-feared Option A. Brown, who has been meeting the president's science adviser Dr John Gibbons, issued a statement last Friday saying that "there will be room for compromise among the differing points of view".

Option C is regarded with suspicion by Freedom supporters because it appears to reverse the entire principle of a modular, expandable and international space station. It also upsets the international partners on the project: by providing much more rack space for scientific experi-

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Japan flirts modestly with international review

Tokyo. The international scrutiny of Japanese laboratories seems to be catching on. Earlier this year, the University of Tokyo opened its physics department to external review (*Nature* 362, 387; 1993). Now, the Institute of Physical and Chemical Research (RIKEN) is following suit. Next week, an international team will visit the laboratory complex to evaluate RIKEN's management and research.

It is not surprising that RIKEN, located in the northwest outskirts of Tokyo, should be the first government-supported laboratory to risk external evaluation. A semi-autonomous corporation supported primarily by the Science and Technology Agency, it is one of the most dynamic and flexible of Japan's research institutes. In recent years, for example, it has been an eager (and imaginative) employer of overseas researchers.

RIKEN's research covers a broad range of science and technology, from the physical sciences and engineering to biology and the medical sciences. It has a branch for life science research at Tsukuba and (with the Japan Atomic Energy Research Institute) is building the world's most powerful synchrotron at 8 Ge in Harima science garden city, west of Osaka. RIKEN also recently opened a laboratory for photodynamic research at Sendai, north of Tokyo, and another at Nagoya for research on biomimetic control as part of its Frontier Research Programme, some of whose project leaders are from overseas.

RIKEN plans to go further in its own evaluation than the University of Tokyo, which has not yet decided whether the external review of physics will be repeated, let alone extended to other departments. Before knowing the outcome of the first external review, RIKEN says the process will be repeated every two years.

The evaluation will be done by what is called the RIKEN Advisory Council (RAC), whose 15 members include Nobel prizewinner Heinrich Rohrer of IBM's research laboratory in Switzerland, Lord Adrian, master of Pembroke College at the University of Cambridge in Britain and Thomas Everhart, president of the California Institute of Technology in the United States. They are joined by scientists from Japan, including Setsuro Ebashi, former director-general of the three Okazaki national research institutes, Michiyuki Uenohara, a former senior executive of NEC Corporation, and Tokindo Okada, emeritus professor of Kyoto University.

The council will be briefed on RIKEN next Monday [21 June], after which five subcommittees will spend three days examining different laboratories. Its report will first go to RIKEN's president, Minoru Oda, who

has keenly supported the idea of an external review, first put forward in 1990 by George Clarke of the Massachusetts Institute of Technology.

RIKEN is by now well used to evaluation. Since 1983, there has been a regular cycle of reviews by teams of four outsiders, three from Japanese universities and national laboratories and one from industry. Each laboratory is dealt with every seven years or so, and the results often stimulate action, even reorganization. But RAC will evaluate the research programme as a whole as well as the management of RIKEN, the policy of the board of directors included. With the exception of the one-off review of Tokyo University's physics department, procedures of this kind are unheard of at other Japanese government research facilities.

But other national laboratories are soon expected to follow RIKEN. The Agency of

Industry, Science and Technology of the Ministry of International Trade and Industry (MITI) has since last year been discussing an international evaluation of its research institutes at Tsukuba, but has encountered considerable resistance among some researchers. MITI officials are nevertheless confident that a system will be operating in this fiscal year, perhaps by October.

It is therefore all the odder that all has gone quiet at Tokyo University since Akito Arima, who mounted the external review of the physics department, retired as university president at the end of March. His successor, Hiroyuki Yoshikawa, has made no public statements on the matter, while the university's public relations department says that "the future of the external review process is unknown at present". Even the university departments now discussing the possibility of external reviews know that they do not have the money to pay for them, but there is also resistance from some faculty members to the mere idea. The moves by RIKEN and other national research institutes may help stimulate Tokyo and other universities into action.

David Swinbanks

China's science budget outgrows economy

Beijing. China will this year increase spending on science by more than 10 per cent in recognition of its potential contribution to the national economy, the fastest growing in the world.

The 1993 budget endorsed last month by the Eighth National People's Congress allocates almost 20 billion yuan (US\$3.5 billion) for science and technology and 60 billion yuan for education. Despite a projected deficit of 3.5 billion yuan, the budget assumes an annual growth rate of 9 per cent. (Other sources estimate an increase of as much as 13 per cent.)

Science is a major focus of the budget. Although spending figures on individual projects are regarded as a secret (see *Nature* 362, 685; 1993), Zou Jia-Ha, vice-premier and director of the State Planning Commission, mentioned five areas where the government is increasing its investment in sci-

ence in a speech to the congress.

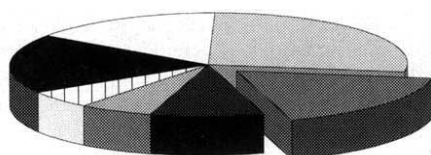
The government has selected 600–800 projects, many involving microelectronics, where it expects to translate basic research into commercial products. At the same time, Zou said, existing state initiatives on applied technology such as the Climbing Programme, the 863 High-Technology Programme, the Torch Programme and the Sparkle Programme will be fully funded. The government has also proposed building a network of state laboratories, ecological facilities and engineering centres, and has promised to make existing laboratories and research centres more efficient. Finally, undergraduate enrolment will be increased, as will the number of universities.

Although the increased budget is welcome, some regard the increases as insufficient. Hu Jian, an official at the National Natural Sciences Foundation, China's main source of funds for basic science, says that an increase in its budget of 20 per cent a year over the next three years will still not satisfy demand. In education, deputies to the recent People's Congress demanded that the government pay teachers on time — salaries are often withheld for as long as six months.

The government says the research will strengthen the economy, "to make research, trade and economy all in one". This approach, underlined by the recent forced realignment of 70 per cent of the scientific staff at the Chinese Academy of Sciences towards applied research, naturally moderates the enthusiasm of Chinese scientists for the news of more money for science.

You Qin Li

1993 science budget



■ Science, health & education
 ■ National defence
 ■ Capital construction
 ■ Agriculture
 ■ Price & debit subsidies
 ■ Industry
 ■ Admin. & other costs

UK rejects call for more systematic biology cash

London. Despite claiming a growing awareness of the need to preserve global biodiversity, the British government has rejected demands from the House of Lords that it should provide extra funding for research in systematic biology, the basic discipline which underpins such efforts.

Eighteen months ago, the science and technology committee of the House of Lords issued signals with a strongly worded report warning that systematic biology — the classification of living organisms based on their evolutionary relationships — was in a state of crisis (see *Nature* 355, 488; 1992).

The committee pointed out that there had been no increase in funding during the 1980s, that research council funding had fallen by a third over this period, and that research staff in the museums responsible for maintaining national collections of biological materials had dropped by 25 per cent.

In its response to the report, published last week, the government's Office of Science and Technology (OST) says it endorses the importance of systematic biology as a discipline underpinning its policy initiatives on biodiversity and the environment, and accepts several of the committee's conclusions.

For example, OST has promised to carry out a review of microbial culture collection in Britain to see how these meet national needs, and how a strategy for the future might be developed. It would like to see increased funding for systematics research in the next Framework programme of the Commission of the European Communities.

The government has also agreed to fund a national forum bringing together the main public and private institutions working in the field. The purpose, it says, will be to disseminate information and "good practice" among the bodies which maintain sizeable systematics collections, and to strengthen links with overseas institutions in order to build up an international framework for the subject.

But there will not be any more money — or even any firm commitments to maintain funding at current levels. The Lords committee had proposed that the research councils be given an extra £1 million a year over the next five years to make good the previous decline. However the OST says it agrees with the Advisory Board for the Research Councils that there is no case for giving systematic biology any special treatment.

Similarly, the government rejects the committee's suggestion that core funding for both systematic biology research and the national institutions where much of it is carried out be maintained at current levels. It says that earmarking resources in such a way

"would not allow museums the freedom to target areas which, in their assessment, warrant priority action."

The lack of any new funding has come as a disappointment to the scientific community. "Even with current funding difficulties, people had been hoping for something a little more positive than this," says Michael Claridge, professor of entomology at the University of Wales College of Cardiff, who acted as an adviser to the Lords committee. "The government's reaction is bound to have a deadening effect."

The reaction of museums, already facing the possibility of further cuts in their operating budgets, has been similar. Neil Chalmers, the director of the Natural History Museum — which, as one of Europe's main centres of systematic biology, is already offering to host the national forum which the government has promised — points out that the museum is finding it

increasingly difficult to maintain core funding for systematic research at a time when its government grant is being reduced.

Government officials point out that the House of Lords report has already helped to focus the attention of the scientific community on the difficulties facing systematic biology. For example, the Natural Environment Research Council (NERC) recently awarded new grants totalling £2.5 million over five years to support taxonomic research and training relevant to the environmental sciences at three British universities (see *Nature* 362, 383; 1993).

But there is widespread feeling that the efforts remain inadequate to the need. The NERC money, for example, will be able to support only short-term appointments, whereas the main need faced by universities is how to fill the senior academic posts that will become vacant over the next few years when their current occupants retire.

Similarly, while the government continues to claim credit for having allocated £6 million over the next few years to back up its commitment to biodiversity, Lord Dainton, the chairman of the House of Lords Committee, describes this as "a complete mouse" compared to the needs. **David Dickson**

Hillary Clinton lobbies at Hopkins for health

Baltimore. Hillary Rodham Clinton, whose husband, US President Bill Clinton, made her head of his task force on health care reform, took her message to US academic researchers for the first time last week, at the centennial celebration of the Johns Hopkins University School of Medicine. Addressing an audience of research physicians, academic scientists and staff at one of the elite medical institutions of the United States, Mrs Clinton declared that what the country needs most is a larger corps of general "primary care" doctors to work in small towns and inner cities, attending to routine medical needs.

The debate on primary care versus specialism is at least a decade old. Mrs Clinton said last week that if medical schools did not change the curriculum to induce more students into primary care, the government would force the issue, perhaps by offering "forgiveness" of educational loans to doctors choosing general medical practice.

Mrs Clinton also suggested that the health reform package would include a proposal to alter or eliminate the capitation funds that medical schools receive by law from the federally run Medicare system. As things are, the government recognizes an obligation to reimburse hospitals for the care provided by medical residents in training at US teaching hospitals. Cutting the Medicare allowance would save the government an estimated \$12 billion a year — or put teaching hospitals in debt by the same amount.

While praising Hopkins for its accomplishments in research and successful

high-technology medicine, Mrs Clinton warned academic hospitals that they must find ways of extending their expertise more broadly into the community. She said, for example, that more attention should be paid to information networks that would enable a small town physician to send a CAT scan electronically to an academic centre where a specialist could read it on a monitor.

Mrs Clinton also paid lip service to funding for basic biomedical research when she said, "We will look for ways to support it" (perhaps not intending to imply ignorance of the US National Institutes of Health), but then closed with an admonition to doctors of all stripes to spend more time talking to their patients about diet, nutrition and exercise, while also "cooperating" more effectively with nurses and each other.

Despite its modest content, Mrs Clinton's speech was well received and won praise for the professionalism of her delivery. The Clintons' health care task force has been notable for the groups that have been deliberately left out of the planning on the grounds that they constitute a special interest and are, therefore, unable to offer objective advice.

That the White House accepted Hopkins' invitation to Mrs Clinton may, in the circumstances, be taken as a sign that the research community has been recognized as an important constituency in the debate on the reform of health care. Despite the president's best intentions, that is unlikely to end soon. **Barbara. J. Culliton**

Lack of vision weakens links between industry and finance

London. Financial institutions in the City of London reject the charge that they are largely to blame for reluctance to invest in industrial research and development in Britain. Equally guilty, financial analysts argued last week, are industrial managers who fail to appreciate the importance of technological innovation and government officials who have been unable to provide a sufficiently stable economic environment to encourage long-term planning.

This genteel name-calling was one feature of a meeting held last week in London's Guildhall, at the centre of the City's financial district, by the British Association for the Advancement of Science (BA). But both sides agreed that changes are needed in the working relationship between the financial and manufacturing sectors in Britain if the "partnership" advocated in the government's recent White Paper on the organization of research is to be achieved.

But not every sceptic is convinced by the City's pleas of innocence. Critics such as the Save British Science campaign attribute a lack of enthusiasm for investing in high-technology companies on the conservatism of financial institutions such as pension funds, which assess fund managers' performance by high dividend returns.

Confirmation of the scale of the problem emerged on the same day as the BA meeting from details published in *R&D Scoreboard*, an annual report on R&D spending by British companies produced by the Edinburgh-based consulting group Company Reporting Ltd and sponsored by the Department of Trade and Industry.

The good news is that such spending, which declined during the recession at the end of the 1980s, is now on the increase. Although British industry's profits continue to fall, its R&D spending rose by 6 per cent last year, with the biggest increases in the general manufacturing sector (23 per cent) and health care (at 16 per cent, stimulated in particular by a 25 per cent growth in the R&D budget of Glaxo, the pharmaceutical company).

More sobering, to use the description of Michael Heseltine, the president of the board of trade, in his introduction to the report, are two other statistics. First, the global increase in R&D spending, averaging 8 per cent for the world's top 200 companies, was even higher than in Britain.

Second, the average spending on R&D by the top 200 companies was 4.6 per cent of sales, compared with a meagre 1.6 per cent in the United Kingdom. Furthermore, in stark contrast to many large interna-

tional corporations, British companies distributed about five times as great a proportion of their profits as dividends as the global average.

Further evidence of a structural link between a lack of long-term perspective and industry's failure to acknowledge the central importance of science and engineering comes in a study published this week by the Economic and Social Research Council.

The study, carried out by Derek Bosworth of the Manchester School of Management and Robert Wilson of the University of Warwick's Institute for Employment Research, shows that the greater the number of graduate scientists and engineers in a company's senior management, the more likely is the company to have formal management goals — and to outperform companies that lack them.

Bosworth claims that the research provides the first clear evidence that individuals with a science and engineering background are important to a company's long-term growth and profits. "They are prepared to wait longer for a payback from investment rather than concentrate on short-term profits," he says.

Technical people may also be more effective in stimulating what Robert Malpas, the chairman of Cookson Group and a former managing director of BP (the petroleum group), identified at the BA meeting as a key factor missing in many companies: the ability to stimulate a "demand pull" for technology. Noting that the government had rejected the idea of new institutions that might have encouraged such thinking, Malpas said that he had "doubts" about the wisdom of its decision.

Another need, according to David McGeekin, corporate finance director of the Midland Bank, is for new mechanisms to encourage financial institutions to invest in high-technology companies, particularly small and medium-sized enterprises (SMEs) too young to have a track record of success.

In the past, he acknowledged, the City's response to requests for finance from these companies had been "rather poor"; financial institutions and bankers would have to develop a new range of instruments that would more accurately reflect the risk-reward ratios in the SME sector.

Others at the meeting argued that there is still an inherent reluctance to take the risks needed to help small companies get off the ground — typified by the restrictive rules applied by the Stock Exchange to new companies seeking a public listing.

At least one research council is taking steps to deal with this problem head-on. Senior officials at the Medical Research Council say they intend to ask the Stock Exchange how its rules might be modified to make it easier for small biotechnology companies to raise money from British investors and institutions. **David Dickson**

Britain's top 10 R&D spenders are dwarfed by global giants

Company	R&D spend 1992 (£million)	% Increase over 1991	R&D as % of dividends
Imperial Chemical Industries	647	9	164.6
Glaxo	595	25	116.2
Smithkline Beecham	478	11	179.0
Unilever	461	8	98.5
Shell Transport and Trading	435	7	19.7
General Electric	417	-4	161.6
BP (British Petroleum)	315	2	55.5
Wellcome	255	11	227.5
BT (British Telecommunications)	240	-1	27.0
Rolls Royce	229	6	477.1
All industry composite	6,475	6	48.3
Top 200 companies worldwide			
General Motors (USA)	3,908	1	550.5
Daimler-Benz (Germany)	3,797	11	1,541.7
Siemens (Germany)	3,419	6	1,153.4
IBM (USA)	3,357	2	183.8
Ford (USA)	2,861	16	443.2
Hitachi (Japan)	2,749	6	1,422.2
Toyota Motor (Japan)	2,365	4	664.5
Matsushita (Japan)	2,212	9	1,452.0
Fujitsu (Japan)	2,073	19	2,059.0
AT&T (USA)	1,923	-7	165.5
All industry composite	103,939	8	257.8

No easy route of evolution for weapons labs, says OTA

Washington. What is to happen to the atomic weapons laboratories in the United States now that the Cold War has ended? The best way forward for the laboratories, mostly controlled by the Department of Energy (DoE), would be for them to follow a broad spectrum of collaborative research aimed at improving US industrial competitiveness, according to a report prepared by the congressional Office of Technology Assessment (OTA).

OTA in particular insists that none of the laboratories could be adequately occupied by large individual programmes, such as the Clinton administration's plan to help develop a clean car. Such schemes, however ambitious, could occupy only a small proportion of the resources of the vast Lawrence Livermore, Los Alamos and Sandia national laboratories, which have a combined budget of \$3.4 billion and employ 24,000 people.

OTA's report comes at a time when several more pieces of legislation assigning new responsibilities to the laboratories have been launched in the Congress, and when uncertainty among members of the laboratories' scientific staffs is growing.

Bending the national laboratories to civil uses has been on the open agenda for several years. Several collaborations with industry are already under way. OTA now argues that the laboratories would be better placed to generate more work through cooperative research and development agreements (CRADAs) if the DoE were to speed up the process of authorizing them: on past form, it takes six months for an agreement to be approved. So OTA suggests that the department's bureaucracy should be streamlined, and pleads for a better understanding by Congress of how legislation, even in apparently unrelated fields, can impede the process.

Despite the virtual cessation of nuclear weapons development, Livermore, Los Alamos and Sandia are said to be still chiefly orientated towards other nuclear work, such as weapon maintenance and clean-up, says OTA senior associate Julie Gorte. "It has been horrendously difficult for industry to do collaborative research with the Department of Energy labs", she says.

Department of Energy laboratories have signed almost 400 CRADAs in the past two years, and within the department's Defense Programs division they have a budget of \$141 million. But that is still a small portion of the laboratories' total budgets.

OTA also suggests that Congress, when framing legislation, must make up its mind exactly what it wants to achieve in the exploitation of new knowledge arising from

work at the national laboratories. At present, industrial collaborations are impeded by legal requirements that products emerging from collaborative work at the national laboratories should preferably be manufactured in the United States. Restrictions on the disposal of property rights are given as another cause of difficulty.

OTA points out that clauses binding collaborators to US manufacture, frequently inserted in US research legislation (see *Nature* **363**, 481; 1993), will deter many participants and therefore reduce the prospects of saving jobs at the DoE laboratories. Similarly on intellectual property, a choice must be made between the wish to disseminate knowledge widely, on the one hand, and to attract partners who want to safeguard it for themselves, on the other.

The OTA report is published as bills

progress through Congress with the aim of providing a new mission for the DoE laboratories. The Laboratory Technology bill being discussed by Science, Space and Technology Committee in the House of Representatives and the Department of Energy National Competitiveness bill proposed by the Senate's Energy Committee are likely to be reconciled with each other and passed later this summer. They already include some of the changes suggested by the OTA, such as the delegation of the authorization of small collaborative projects directly to the laboratory directors.

The OTA examined two large initiatives which had been mooted as potential 'saviours' of the weapons laboratories — the development of clean cars and of high-technology public transport systems — and concluded that these or other such programmes could have only a limited impact.

Energy secretary Hazel O'Leary welcomed the OTA report and promises to "respond with vigour". "I have set several efforts in motion during the past few months to help put our technology transfer house in order," she says.

Colin Macilwain

UK to close environmental research lab

London. Scientific staff of Britain's main research centre for environmental technology, the Warren Spring Laboratory at Welwyn outside London, are trying to block the government's plans to merge the laboratory with AEA Technology in Oxfordshire, a move that is likely to lead to the loss of up to 150 jobs.

This move follows an announcement by Michael Heseltine, who as president of the board of trade is head of the Department of Trade and Industry (DTI) and thus the government minister responsible for both organizations, that they are to be combined into a single centre of excellence known as the National Environmental Technology Centre (NTEC).

Heseltine told Parliament last week that the goal of the merger is to combine the complementary strengths of the two organizations, ensuring a "more comprehensive service" for their customers and better value for taxpayers's money. AEA Technology already has a large group of researchers working on environmental issues.

But the Institution for Professionals, Managers and Specialists, the trade union representing more than 200 scientific staff at Warren Spring, argues that the merger is the direct result of the DTI's decision to reduce its spending on research. At risk, it says, are a number of important research projects, such as several into techniques for monitoring air pollution, which would not easily attract sponsorship from the private sector.

Union representatives have been promised a meeting with Heseltine this week to express their concern. They are planning to

tell him that his decision contradicts a promise made to Parliament last month that the future of the DTI's five research laboratories would be decided only after the completion of a study into their future prospects by the consulting firm KPMG.

They are also planning to mount a legal challenge to the DTI's plans to transfer staff from Welwyn to AEA Technology, claiming that Heseltine lacks the authority to require such a move.

DTI officials deny that the decision on Warren Spring is premature, claiming that the merger does not amount to the closure of the laboratory — and that the KPMG survey will be used to assess whether the NTEC should be privatized (a path to which AEA Technology is already committed).

The move of research teams to Oxfordshire will, however, be a considerable embarrassment to the government. Only last year, the then trade minister Peter Lilley announced plans to erect a new building for the Warren Spring Laboratory in Welwyn, at a cost of £25 million. The site of the new building has already been prepared, at a cost of £7 million, and this money will have to be written off.

The government decision to merge the two laboratories has also been criticized by the opposition Labour party. Jim Cousins, the party spokesman on trade and industry, claimed last week that there will inevitably be tensions between an organization that has been devoted to research into ways of protecting the environment and another whose traditional role has been to promote the development and use of nuclear power.

David Dickson

Profits of British science-based companies boom

London. Underlining the potential links between science and wealth creation in a way that will warm hearts in the Cabinet Office, two British science-based companies last week reported significant increases in profits.

Amersham International, the health science group that grew out of the radioisotope supply service of the UK Atomic Energy Authority, announced that profits had increased by 27 per cent to £26.3 million in the financial year ending 31 March. And at Oxford Instruments, the advanced instrumentation group, profits have risen by 24 per cent to £10.6 million, thanks largely to the growing success of a joint venture set up with the German company Siemens to market nuclear magnetic resonance imaging (MRI) equipment for body scanning.

Amersham was one of the first government agencies to be privatized by the former Prime Minister Margaret Thatcher in the early 1980s. It has since become the world's leading supplier of reagents for genome sequencing, boosted by its recent acquisition of US Biochemicals Corporation. Strong sales to medical and pharmaceutical research laboratories in both the United States and Britain resulted in an increase in turnover by its life sciences division from £92.7 million to £98.9 million.

The profits of this division nevertheless fell slightly, to £19.1 million, partly because of a flattening of sales in mainland Europe and Japan. But this fall in profits was also due to a 24 per cent increase of the division's spending on research and development, partly reflecting the company's efforts to develop new products for DNA sequencing. Later this year, for example, the company plans to launch what it calls a "laboratory robot" for purifying and sequencing DNA.

Like Amersham International, Oxford Instruments — a welcome turnaround after two successive years of declining profits — has benefited from the fact that a large proportion of its sales are outside Britain, so that the company benefited from the devaluation of sterling last autumn.

Future prospects for Oxford Instruments will depend crucially on whether impending health reforms in the United States curb the potential market for body scanners. The company is also taking a chance on its ability to find a customer for a second synchrotron machine, designed to increase the density of circuits on microchips, which is under construction and will eventually be offered at a price of about £16 million (\$25 million); the first such synchrotron built by the company is now being used almost exclusively by IBM.

David Dickson

Stanford seeks life after Cohen-Boyer patent expires

San Francisco. Stanford University in California is seeking a bridge across the gap in its finances that will be created when the Cohen-Boyer patent expires in 1997.

The patent, which covers insertion of foreign genetic material into bacterial plasmids, will by then have earned about \$87 million — an amount that has risen steadily each year since 1980 and which last year reached almost \$15 million. The royalties are shared with the University of California (UC) because Boyer is a professor of biochemistry and biophysics (now emeritus) at UC-San Francisco.

members hardest, as they share two-thirds of a patent's royalty income. David Botstein, chair of Stanford's department of genetics, says that the Cohen-Boyer money has helped to pay for new laboratories and renovations and to attract new faculty. But he compares the impact of the money to that of a legacy to a prudent working person: "they wouldn't make their lifestyle dependent on it".

Katharine Ku, director of Stanford's Office of Technology Licensing says that research grants from the federal government, nonprofit organizations and industry provide much more money each year —

some \$300 million — and are a more reliable source of funding.

Nevertheless, Stanford hopes to make good the loss, following recommendations of an alumni task force, by taking equity rather than royalties from outside companies licensing inventions. Taking the title to all inventions involving the use of Stanford facilities or resources, including inventions funded by gift money, would also help.

The alumni also suggest that Stanford should clarify its policies on the work done by individual faculty members for outside partners, for fear that patenting policies may founder on conflicts of interest. The faculty has not yet reacted to that proposal, nor to the suggestion that Stanford should concentrate on technol-

ogy with the highest potential return.

As a token of willingness to be more active on technology licensing, the university has transferred \$2 million to its Research Incentive Fund, which provides faculty with development seed money.

Ku, whose office operates on money from a 15 per cent share of licensing revenues, is hopeful about inventions for sound synthesis for multimedia products, as well as about a fundamental technology for atomic resolution instruments and memory systems. His personal choice is nanotechnology.

In any event, Stanford has learned that patents are a long-term and difficult source of funding. Nevertheless, after subtracting payments to other universities, Stanford last year received \$18.5 million. **Sally Lehrman**

Stanford's royal revenue producers

	Expiration date	Amount
Cohen/Boyer recombinant technology	1997	\$14,660,699
FM sounds	1994	2,712,522
Fluorescent conjugates for analysis of molecules and cells	1992	2,023,125
Computer X-Ray section scanner	1994	1,079,579
Stanford T-shirt logo	TM, no expiration	245,926
Amplification of eucaryotic genes	2004	289,043
Mouse anti-human hybridomas	not patented	287,883
Anti-Leu-12 monoclonal antibody	not patented	230,453
Pisces (software)	not patented	227,415
Antibody to the J-5 fragment of bacterial endotoxin molecule	patent pending	176,821
Variable rate selective excitation pulses for magnetic resonance imaging	USA: 2005 Europe: 2008	171,000
Cell analysis system	expired 1991	157,331
Method of treating autoimmune diseases cell-mediated by Leu3 phenotype T cells	2004	150,000
Contour-clamped electric fields for the separation of macromolecules	2009	145,576
A new approach to digital reverberation	2008	144,990
V-System (software)	not patented	124,025
Asymmetric epoxidation process	2003	111,779
NFAT transcription system for screening the immunosuppressive drugs the Fgl-5 cell line	not patented	111,000

Source: Office of Technology Licensing, Stanford U., 1991-2

Stanford was for many years the most successful academic licensor to private industry in the United States, grossing \$25 million last year, although it now ranks second to the UC system. UC, which draws from a much larger research pool, is enjoying a burst of royalties from maturing patents and expects an additional boost in the mid-1990s from the activities of a recently expanded licensing group (see *Nature* 362, 395; 1993). The Cohen-Boyer patent is not even UC's largest revenue producer, having been eclipsed by a hepatitis vaccine and a nicotine patch.

But at Stanford, the biotechnology patent still reigns supreme (see table). The loss of income from it and several other lucrative patents will hit departments and faculty

Japan embarks on an academic computer network

Tokyo. In an unusual display of cooperation, Japan's science-related ministries and agencies are trying to work together to build a high-capacity computer network linking universities and national research institutes, providing them with access to scientific computer networks outside Japan. But it remains to be seen whether the planned network can bypass the bureaucratic walls that traditionally divide Japan's government research organizations.

Last week, ten Japanese officials from the Science and Technology Agency (STA) and the ministries of International Trade and Industry, of Education, Science and Culture and of Posts and Telecommunications visited Washington and New York to study computer networks in the United States. The outcome will be used to draw up plans and budget requests for submission to the

Ministry of Finance in August.

Japan's computer networks are "five years behind the United States", says Tateo Arimoto, director of STA's science and technology information division, who took part in the visit. Whereas the current capacity of NSF's backbone network is 45 megabits per second, Japanese networks operate at less than one hundredth of that speed and are in a much more primitive state of development.

The Japanese government has been very slow off the mark. Most of the initiative for developing networks has been that of individuals. In 1989, Tsuneyoshi Kamae and other researchers at Tokyo University established the Todai International Science Network (TISN) with a 64 kilobit-per-second link to the United States. That has since expanded to other institutes and universi-

ties, but without government backing. Similarly, the Widely Integrated Distributed Environment (WIDE) network for research and development of computer networks was established by Jun Murai of Keio University (*Nature* 356, 550; 1992).

The National Centre for Science Information Systems (NACSIS) under the education ministry has been responsible for linking universities and establishing local area networks on university campuses. But some of the branches of this network have very low capacity, some parts still use a communications protocol that is incompatible with international systems, and only a handful of Japan's 98 national universities have local area networks.

Last month's supplementary budget to stimulate the economy provided a welcome influx of new funds to buy hardware both at universities and national research institutes (see *Nature* 363, 5; 1993). The officials in the United States last week now hope to win more money in next fiscal year's budget to link newly purchased computers and local networks with the proposed new network.

But details of the network remain vague. The financial newspaper *Nikkei Shimbun* reported last week that there will be 30 access points or "hubs" at various locations throughout Japan and that the government will invest 50 billion yen (\$450 million) in the network over the next three years. But Arimoto says the number of hubs has not been fixed and that the budget has been over-estimated, despite the higher cost of domestic telecommunications in Japan than in the United States.

The network's capacity will be limited by the maximum capacity of Japan's domestic carriers, currently 6 megabits per second. But that may be upgraded to 45 megabits in the near future, Arimoto says.

Also to be resolved is the nature of the network's international connections. Kamae, who also heads an advisory committee to the government set up by the Japan Information Centre of Science and Technology under STA, favours for the international link a "neutral" access point in Tokyo not under the jurisdiction of any particular ministry or agency. The node, he says, could consist of an optical fibre loop of about 1 km radius allowing access to any of Japan's competing communications carriers.

Arimoto says that operation of the network should "ideally" be overseen by one organization funded by the various science-related ministries and agencies. But it seems likely that the ministry of education will try to maintain as much control as possible over the university part of the network.

The project has the backing of the ruling Liberal Democratic Party (LDP), which is trying to create a new category of budget for such infrastructure projects. But the Ministry of Finance is strongly resisting the suggestion, meaning that the network may have to be built out of existing resources.

David Swinbanks

Indo-US plutonium dispute flares up

New Delhi. India is heading towards a major diplomatic conflict with the United States over the reprocessing of spent fuel from the US built nuclear power plant at Tarapur, north of Bombay.

The Indian Atomic Energy Commission (AEC) wants to recover plutonium from the spent fuel to use in the Tarapur plant, which no longer gets enriched uranium from abroad. But an official at the US Embassy in Delhi has said that his government will not allow reprocessing unless India signs the nuclear non-proliferation treaty (NPT).

Spent fuel from the two 210-MW boiling water reactors has been accumulating since 1969, and is estimated to contain between 1,000 and 2,000 kg of plutonium. AEC says it belongs to India under the terms of the 1963 Indo-US agreement on Tarapur, and that India will be free to reprocess it after the agreement expires on 25 October. But the official said that permission would still be needed, and is unlikely to be granted under existing [US] law.

Tarapur has soured Indo-US relations since 1974, and the Indian nuclear test at Pokhran. Supplies of enriched uranium fuel were erratic after 1974, and were stopped completely in 1982, when the United States invoked its Nuclear Non-proliferation Act 1978, which prohibits the sale of fuel to countries that are not signatories to NPT. France took over the supply of fuel in 1983, but now says that it will not continue after October unless all Indian nuclear facilities are opened for international inspection. The Indian government is willing neither to sign the NPT nor to accept the French conditions

of full safeguards. It has also ruled out closure of the Tarapur plant, which is a major source of electricity to the power-starved states of Gujarat and Maharashtra.

The AEC says that the Tarapur reactors can be safely run for another 15 years with home-made alternative fuel: an oxide mixture of plutonium and natural uranium, or MOX. It has already set up a manufacturing facility and is waiting only for plutonium to come from its reprocessing plant. The US stand on reprocessing has now upset the calculations.

India's legal right to reprocess spent fuel is ambiguously defined by the 1963 agreement, which gives India ownership of the spent fuel and allows for its reprocessing and reuse at Tarapur. But another clause says that reprocessing requires a "joint determination", a phrase interpreted by the United States as "prior consent". US officials in India say that consent has not previously been given and will not now be given under the Nuclear Non-Proliferation Act.

The AEC, on the other hand, says that "joint determination" means only "approval of the design of the reprocessing plant from the point of safeguardability." In fact, according to AEC, the reprocessing plant was completed "at considerable cost" in 1975 only after the United States had agreed the design. But, despite reminders, the United States failed formally to complete the 'joint determination' exercise, thereby nullifying the crucial clause. In any case, Indian officials say, all obligations under the agreement became unenforceable after the expiry of the agreement.

K. S. Jayaraman

Visit Russia, help science

SIR — Scientific journals regularly describe the continuing decay of Russian science. The cumbersome, highly redundant Russian scientific system has indeed collapsed with the Soviet Union. Although only a few research laboratories have been closed, the contraction of the scientific enterprise is inevitable, despite the traditional belief that a scientist has a job for life. Today, Russian scientists are underpaid, they have inadequate working conditions, they must endure hardships in everyday life and, as result, they leave science or the country.

Does this mean that Russia does not need scientists and that Russian science will eventually cease to exist? The answer is definitely no. Russias badly needs scientists and science to become an integral part of the developed world. Without science, a modern society cannot provide a secure and decent life for its people.

Fortunately, the present situation is not as desperate as it might seem. There are still scientists who work effectively and have decided to stay in Russia. Bright students are still attracted to science. There are still places of excellence, research laboratories and institutes with a solid international reputation in which good science is done. They can serve as focal points for the recovery of Russian science. But they are in urgent need of help. Scientists must have access to the literature, reagents, equipment and international meetings and, of course, they must have adequate salaries. And help is coming through Russian government scientific programmes, sometimes from their own commercial activities, and, essentially, from international grants and contracts with foreign companies and Western foundations.

But Russian science will inevitably shrink and only the best should remain. Who will survive? These decisions should not be made only by the government or by the academic hierarchy. If they are, the choices may be based on political considerations rather than on the quality of the science. The research enterprise must undergo quality selection, like its counterparts in the West. Research groups will have to take a much more aggressive approach to survival. They must compete for support, for grants and for scientists of the highest calibre. The vacancies created by the devastating brain drain from the best Russian institutions should be filled from both inside and outside the country to keep science running. The feudal system within which most Russian scientists spent their entire careers as subordinates in just one place is dying, for better or worse. A demand for scientists will develop. In the near future, advertisements for positions in Russian research centres

are likely to appear in international journals.

The situation at the Engelhardt Institute of Molecular Biology is no exception: we too are struggling for survival. The institute is a leader in molecular and cell biology and in human genome studies. But, like many other institutes, we have recently endured a period when we found it very difficult to maintain scientific productivity. Fortunately, the institute has sufficiently good facilities and equipment to be internationally competitive and it has succeeded in retaining a core of outstanding scientists. They have continued to work productively and, surprisingly, the number of publications in leading international journals has continued to increase. We have maintained our traditions of scientific openness, cooperation and independence. The institute actively participates in several state scientific programmes; for example, it is the leading institute in the Russian Human Genome Project. We maintain close contacts with many Western laboratories. All this has proved useful in winning Russian and international grants to cover research expenses and salaries.

However, the institute has not escaped the impact of the brain drain. We now have a number of positions available at various levels, ranging from technicians to laboratory heads. We are inviting both Russian and foreign researchers to apply for these positions, which have a tenure of 2 to 5 years. On request, we shall send a brochure about the institute. A sojourn in Russia is both challenging and attractive.

Andrei D. Mirzabekov

*Engelhardt Institute of Molecular Biology,
Russian Academy of Sciences,
Moscow 117984,
Russia
Fax (7095) 135 1405*

Problem-solving

SIR — In various places (for example *Nature* 361, 571; 1993), you have expressed your views concerning the (re)organization of British science. You conclude that academic research should be primarily educational, and that education should be in problem-solving. Thus far, I, an industrial scientist, am in agreement.

Then we part company. You hold that apprenticeship in production management does not teach problem-solving. You would be right if plant and factory ran as smoothly as they should. As they never do, managing them demands problem-solving of an ingenuity seldom shown in academic life under pressures professors have never dreamt of. Production mana-

gers, very properly, detest innovation — they have learnt that the state of the art is down and that infant technologies are always unable to compete with the mature ones they will grow to supplant.

My colleagues and I and my more reflective academic friends have long been alarmed at the increasing number of production lines in academic research, in big science and elsewhere. There are many projects that consist of tedious repetition of one simple experiment to amass data which, combined with those from numerous similar slave labourers, might conceivably solve a problem some decades hence. The stamp collectors of high-energy physics are at this game and it is a fair description of the gene-sequencing at the heart of molecular biology. We are meeting potential recruits with doctorates in handle-turning and machine-minding.

If you were to ask my managerial colleagues who were their best problem-solvers in the 25–30 age group, they would not say the young PhDs. Their answer would be the technicians who have done part-time degrees while working in our research laboratories. For this there is another reason. If it can be solved in the library, or merely by taking thought, it is either no problem or not research. Problems, the innate malice and perversity of matter and men, must be fought hands-on and with sleeves rolled.

The average academic now ceases to engage in hands-on research shortly after the age of 30, thereafter becoming a part-time administrator of research students. At this age, the industrial PhD is still learning his craft, full-time without teaching duties. This he will eventually pass to the next generation by example, not by lecture-hall harangue.

The result is that the real expertise in, say, synthetic organic chemistry is not in the Universities of Nottingham or Cambridge, to name two highly praised exemplars, but in the pharmaceutical research divisions of industry. Fortunately for academics, the managerial classes share the general anthropological belief that foreign magic is more powerful than domestic magic.

If we in industry encounter a problem that might or might not yield to a great deal of tedious and repetitive research but is not vital, we commonly look for an academic collaboration. There are always academics looking to fund a mental research and willing to sentence a graduate to it. To my shame, I have sometimes been involved in the supervision of such projects, for which reason I use a pseudonym.

To meet our aims, academic life needs transformation unlikely to be achieved by those who have lived entirely in the present structure.

Simon Roman

*2 Upper Rosemary Hill,
Kenilworth CV8 2PA, UK*

Opposition to Oncomouse

SIR — Your reporting on the European opposition to patents for the Harvard Oncomouse (*Nature* **361**, 103 & 574; 1993) concentrates on the opposition generated because the mouse is “designed for suffering”. That may indeed be a concern, but there are other more important issues involved in the matter of “patents on life”. At least some of the opposition is motivated by matters separate from animal rights.

The opposition to the oncomouse patent is part of a pattern of opposition to the patenting of any form of genetically altered organism. Organisms that have been ‘designed for suffering’ have been with us for some time. They have been developed by ordinary methods of selection. Sometimes the purposes for which they were bred have been rational, for example athymic nude mice. Sometimes they have been irrational, for example the incredibly unhealthy purebred dogs that practising veterinarians such as myself have to deal with.

What is different about the newer form of genetically engineered organisms is that their developers are now demanding the protection of governments for their economic interests. This attempt to use the state to enforce a monopoly means that genetic engineering is now a matter of public interest rather than a private economic or research concern.

Large vested interests are too often assured of the protection of government for the promotion of their own interests. The public surely has a right to question how its tax dollars are spent. The institutions that develop genetically engineered organisms are usually the recipients of large sums of public largesse whether directly or indirectly. If the public's money is being spent, the public has a right to intervene in how it is being spent. This is part of a larger struggle for democracy and participation that is one of the characteristic trends of our time. It should also be noted that allowing an individual or institution to gather further economic benefits from the use of public money seems to violate elementary standards of fairness.

There is also the matter of how the granting of patents on life is situated in our economic system. Practising veterinarians are more than aware of how some aspects of agriculture have become monopolized by larger corporations given the advantages that government already provides to such institutions. Patents on genetically engineered organisms will only encourage this trend to monopoly agriculture. Is this a desirable outcome? Does it not conflict with the desire for more democracy and more individual and local control? Is it right to allow corporations to use the

powers of government further to disempower individuals in our society?

Pat Murtagh

*Mobile Veterinary Services,
700 Henderson Hwy,
Winnipeg, Manitoba R2K 2J8,
Canada*

Wrath averted

SIR — In your Commentary “Forty years of molecular information” (*Nature* **362**, 783–784; 1993) you state that I recalled the biochemistry department from Oxford travelling together to Cambridge to look at the DNA structure. This is incorrect. The group that travelled consisted of Sydney Brenner, Jack Dunitz and myself (none of us from the department of biochemistry). Please correct this to prevent historical inaccuracy, as well as to protect me from the wrath of my colleagues.

Leslie E. Orgel

*Salk Institute,
Chemical Evolution Laboratory,
PO Box 85800,
San Diego,
California 92186-5800, USA*

Opportunistic infection

SIR — You rightly call for “a better understanding of the way in which the existence of a substantial pool of people with serious immune suppression may contribute to the emergence of virulent forms of other infections, tuberculosis for example”¹. In fact, although the contribution to the development of more virulent forms is not yet clear, there are already a number of well-argued cases of AIDS sufferers incubating opportunistic pathogens and then acting as reservoirs and passing these secondary diseases on to others.

Chave *et al.*² have reported five cases of *Pneumocystis carinii* pneumonia (PCP) in renal transplant patients who shared the waiting room and some of the treatment rooms of a Swiss clinic with AIDS patients, as well as an increase of PCP in cancer patients coincidental with increased registration of AIDS cases. Bods-worth, Cooper and Donovan³ have studied the passing of hepatitis-B (HBV) from AIDS patients to others.

There is at least one documented example of transmission of multidrug-resistant tuberculosis from an HIV-positive patient⁴. And Fitzgerald, Grzybowski and Allen have traced the coincident rises of HIV and tuberculosis in an extensively

documented study⁵.

Because the secondary infections which AIDS sufferers incubate are most dangerous to those with weak immune defences⁶, the dangers are greatest in the hospital environment. AIDS sufferers must obviously be isolated from other patients with weak immune defences. The idea of an AIDS ward also becomes questionable because AIDS sufferers are probably most dangerous to other AIDS sufferers.

The dangers to the general healthy community also ought not to be underestimated. For tuberculosis endangers everyone and not just the immunosuppressed.

Frank J. Leavitt

*The Lord Immanuel Jakobovits
Center for Jewish Medical Ethics,
Faculty of Health Sciences,
Ben Gurion University of the Negev,
Beer Sheva,
Israel*

1. *Nature* **362**, 13 (1993).
2. Chave, J. P. *et al.* *AIDS* **8**, 927–932 (1991).
3. Bods-worth, N. J., Cooper, D. A. & Donovan, B. J. *Infectious Diseases* **163**, 1138–1140 (1990).
4. *MMWR* **40**, 129–131 (1991).
5. Fitzgerald, J., Grzybowski, S. & Allen, E. *Chest* **100**, 191–200 (1991).
6. Bronnimann, D. A. *et al.* *Ann. int. Med.* **106**, 372–379 (1987).

One too many

SIR — Daedalus is a strange and mysterious creature, who may well carry 24 pairs of chromosomes¹. Personally I am satisfied with only 23 pairs, a number I share with almost all other humans².

Bengt O. Bengtsson

*Department of Genetics,
Sölvegatan 29,
S-223 62 Lund,
Sweden*

1. Jones, D. *Nature* **362**, 594 (1993).
2. Tjio, J. H. & Levan, A. *Hereditas* **42**, 1 (1956).

Un morceau

SIR — The British Library has purchased the William Petty papers from Lord Shelburne for £1 million. . .

*A million pounds was paid, I read¹,
To satisfy Lord Shelburne's need.
(It would be libellous, I fear,
If “greed” for “need” were written here!)
Sir William's papers, maps and things,
For decades hidden in the wings,
At last will publicly be seen.
The British Library, I ween,
Will soon expose, for our delight,
These works, so long obscured from sight.
It took more cash than I have got
To purchase such a Petty lot.*

Andrew Dale

*Department of Mathematical Statistics,
University of Natal,
Durban, 4001,
South Africa*

1. *Nature* **361**, 4 (1993).

Justice is not egalitarian

SIR — I disagree with the egalitarian concept of justice presented by Benno Müller-Hill in his Commentary "The shadow of genetic injustice" (*Nature* 362, 491–492; 1993). We do not need "laws to protect the genetically disadvantaged". The purpose of laws is to establish a framework in which the force of the state may be used to protect the rights of individuals to life, liberty and property.

Individual rights can be violated only through the initiation of force or fraud by other individuals. This is not the case in Müller-Hill's third and fourth examples, which involve a prospective employee being refused employment on the basis of results of screening for a genetic disease.

The only basis for cooperation between free individuals is voluntary consent. There is no "right to a job" if nobody chooses to hire you. There is only the right to free trade — to take a job if offered one.

Laws that require businesses to employ people whom they would rather not are no better than laws in racist societies that forbid employers from hiring people of certain races who would otherwise be hired. They both violate the right of freedom of association.

Genetic screening is but one criterion in determining the market value of one's labour. Job seekers with genetic disease markers may enhance the value of their services by learning special skills in high demand or by lowering the price they ask for their services to give them an advantage. The biggest barrier to job-seekers with disabilities is coercive legislation that obliges employers to pay the medical insurance of their employees and laws that forbid disabled employees to work at a lower wage.

Müller-Hill offers us a choice between "the values of the Nazis and those of Moses". This is no choice at all. The alternative is not self-sacrifice to the nation or self-sacrifice to God. Man must learn to live for his own sake, neither sacrificing himself to others nor sacrificing others to himself.

Ron M. Kagan

12135 Mitchell Avenue, Apt 345,
Los Angeles, California 90066, USA

NIH funding

SIR — While we all would like to see more funding for the US National Institutes of Health (NIH), significant increases are unlikely in the near future. However, the potential for significant changes exists within the current levels of funding. A disproportionate amount of funding goes to individuals with multiple grants at the

expense of intellectual diversity. As the table illustrates, the average cost of a grant increases with the number of grants.

DISTRIBUTION OF NIH RESEARCH GRANT FUNDS, FISCAL 1991

Grants per person	No. of people	No. of grants	Annual amount	Average amount per grant
9	1	9	\$5,707,025	\$634,114
7	4	28	\$13,579,534	\$484,983
6	19	114	\$32,018,786	\$280,867
5	31	155	\$50,957,134	\$328,756
4	168	672	\$177,215,661	\$263,714
3	729	2,187	\$665,995,895	\$258,800
2	3,593	7,186	\$1,606,293,663	\$223,531
1	15,263	15,263	\$2,940,907,779	\$192,682
Totals	19,808	25,614	\$5,392,675,477	

The data represent funding for all NIH research grants, excluding training grants and contracts. The amounts represent total costs including overhead. (Data courtesy Dr S. Joseloff, NIH Division of Research Grants.)

For every application funded, another must go unfunded. As one scientist collects his n -th grant, investigators only a few points lower in priority score are sent away with no funding. Because of the escalation of grant costs with number, if investigators were limited to a maximum of two grants (funded at the current average for two grants, about \$400,000 a year), 4,000 additional independent investigators could be funded each year. (The table underestimates the extent of funding inequity since it doesn't include NIH contracts, training grants, funding from other federal agencies, companies, endowment funds and so on.)

The academic research institutions in the United States value grants over results. Money brings power. By permitting grant hoarding, the NIH supports this executive reward system, with adverse consequences. Competent scientists are forced out of the system with zero funding. Junior faculty may not get tenure because they can't get (enough?) funding. Smart students, seeing the capriciousness of funding, leave science. Good scientists who become grant collectors are diverted to administration instead of science.

By limiting the maximum number of grants per principal investigator to two, the research establishment could be made healthier and more creative. As is recognized by a system of funding independent investigators, people do their best work when they are closely involved with the pursuit of their own ideas.

Frederick Sachs

Biophysical Sciences Channel Group,
120 Cary Hall,
State University at New York,
Buffalo, New York 14214, USA

Refused entry

SIR — Political interference in the free exchange of ideas and scientists has often drawn criticism from the scientific community. For example, the inability of Israeli scientists to procure visas from

the Soviet Union to attend the 14th International Genetics Congress led to a boycott of the congress and denunciations by many scientists (*Nature* 275, 577; 1978).

More recently, exclusion of South Africans by the United Kingdom (*Nature* 320, 3; 1986) and of HIV-positive individuals by the United States was greeted by protests. In both cases, the organizers saw fit to move the congresses as a result: the first from Southampton to Mainz, West Germany, and the second from Boston to Amsterdam.

Unfortunately, we must now report an additional case of political intrusion. A Cuban PhD student in our department, Gerardo Ferbeyre, was refused a visitor's visa by the United States. The refusal cites section 212(f) of the American Immigration and Nationality Act, "which prohibits the issuance of a visa to any person or class of persons whose entry into the United States is deemed by the president to be detrimental to the interests of the United States".

The visa was required by Ferbeyre to attend the conference "Catalytic RNAs (ribozymes) and gene therapy for the treatment of HIV infection" organized by the National Institute of Allergy and Infectious Diseases from 6 to 13 December 1992.

Ferbeyre had been invited to attend the conference and to give an oral presentation of his work. Although we found it mildly amusing that the US government could feel so threatened by a single scientist, the fact remains that the accusations are specious and scandalous. Indeed, it could be ventured that the decision to refuse this visa would in itself be "detrimental to the interests of the United States" if one assumes that this interest includes either increased understanding of AIDS or the establishment of contacts with the Cuban intelligentsia.

An inquiry to the American Consulate in Montreal was bluntly rebuffed with a response to the effect that the American government does not welcome inquiries on the justification of visa decisions.

Apparently the convergence of political ideals that has taken place recently in the world is not to be interpreted to mean that scientists will be sheltered from arbitrary decisions in the future; however, by speaking out, the scientific community can defend the principle of the unimpeded access to scientific information which is so central to our common enterprise.

John Bratty

Robert Cedergren

Département de Biochimie,
Université de Montréal,
Montréal,
Québec H3C 3J7,
Canada

Give the hunger-striker a break

Hunger-striking is not an honourable way of influencing events, but an expression of frustration. Walter Stewart has given up his fast. His employers (the NIH) might better now be able to be conciliatory.

Bethesda. Walter Stewart, the scourge of data-falsifiers everywhere, broke his 33-day fast last Friday evening. The following morning, it was evident that he had lost weight (a pound a day, he says), but his characteristic conversational style had not changed: he speaks as if there were only exclamation marks for punctuation, and has done so for at least the past quarter of a century.

Stewart was sitting on a couch in the living-room of Dr Ned Feder, his partner and fellow employee of the National Institutes of Health (NIH), assigned to the National Institute of Diabetes and Digestive and Kidney Diseases until their reassignment as from 10 May. Feder had earlier explained that the routine has been the same for the past month; Stewart's wife Nancy would drop him off each morning at 8.30 and "we would work together until 6 p.m. or so". Technically, both were on vacation last week.

"Just what you would expect of them", their critics say. "They blow their vacation allowance on a foolish hunger strike, milking the event for the publicity it will bring them." But that is not the case. For what it is worth, there has not been much publicity; even their friends have been a little embarrassed, not having many hunger-strikers among their acquaintance. But camping out in Feder's living-room is also a kind of deprivation, cutting them off from the ample communications at NIH. Feder explains apologetically that "Eva [his wife] wasn't too pleased, but we've filled the place up with fax machines and photocopiers". No complaint about the vacation time, it seems.

Feder's greeting at the door had been a little formal: "You'll be glad to know that Walter gave up his fast last night, after careful deliberation." There had been telephone conversations earlier, but no suggestion that Stewart was anything but implacable. So why did he give up? Because, they both said (but especially Stewart) that they believed they would establish the point they wished to make with NIH. Stewart says that eleven senators support their case. He imagines that there will be some kind of investigation, and that they will be able to continue with their studies of the scientific literature.

What is their case? It is best described anecdotally. I first met Stewart in 1970, when *Nature* set up an office in Washington and when Feder, an earlier friend, recommended Stewart as a referee for "difficult" papers. Stewart was then fresh from a junior fellowship at Harvard, where he and Feder

(who had been at the medical school) had struck up a friendship. So Stewart came to talk, and seemed interested in the tale of a paper about a material called "scotophobin"; an extract of the brain of a rat which, when injected into the skull of another rat, would transfer to the second the learned skills of the first.

The experiments had been described in great detail. Learning consisted of being taught to run a maze, so that there were statistical problems in telling how the second rat (with scotophobin) differed from the first and from naive animals. But there were also problems in peptide chemistry, to do with the purification of the material. Preliminary accounts of the work had already appeared elsewhere, giving it an air of verisimilitude. But Stewart said without hesitation, "It cannot be true", and he laughed aloud. He promised to send a referee's report, which turned out to be as long as, and much more scholarly than, the original paper. We published both, since when nothing much has been heard of scotophobin.

It is not easy to understand why people like Stewart are so captivated by the bogus. Feder, whose behaviour is the more correct, is easier to place as a conventionally morally upright person, to whom deception (especially in science) gives deep offence. Stewart rather marvels at it, like a person seeing a card-trick for the first time, and in the spirit of "aren't those devils even cleverer than we thought?"

Stewart's next brush with *Nature* began early in the 1980s, when he and Feder sent in a manuscript purporting to describe the motives of the coauthors of John C. Darsee, then known to have concocted data both at Emory University and at Harvard. The objective was to show that the coauthors "knew or should have known" that Darsee was a fraud; the attorneys of three of them certainly read the manuscript in that sense. In the endless iterations of the text, on one memorable Friday afternoon, Stewart called to say that they were withdrawing the manuscript on the basis of the first few pages of an edited version still feeding through the fax machine in London. But when eventually a text was published, it did a public service: people think twice, now, about letting their names be added to manuscripts they have not read.

Jointly, Feder and Stewart have maddened the scientific community by what seems to be their arrogance. But, in reality, they have been most often right in their assertions, which are usually narrowly drawn,

and based only on meticulous analysis. But their most recent venture, the notorious "plagiarism machine", software designed to count common sequences in files representing the published literature, is mistaken. The theft of other people's ideas is a greater offence in science than the copying out of mere text.

While nobody has said as much, their public brush earlier this year with the Civil War historian Stephen Oates seems to have been the trigger for their reassignment, Stewart to the Laboratory of Chemical Physics, Feder to the extramural grants division of their institute. It seems not to be widely appreciated that, until then, their duties included the study of "professional practices among scientists, professional misconduct and the accuracy of the scientific literature". Both men, as it happens, were given ratings of "excellent" at their routine performance assessments in the summer of last year.

What will happen now? Still recovering last weekend from his fast, Stewart seemed most of all offended that neither he nor Feder will be allowed to attend the symposium on plagiarism and its machine detection organized by NIH and the American Association for the Advancement of Science for 21–22 June. Their plagiarism program, already used elsewhere, will be described by others. The NIH evidently believes it is a broad church, within which it has hitherto accommodated ingenious Feder and Stewart. Why not carry on a little longer?

Evidently there is a practical problem. Feder and Stewart make waves which, when spurious, are more than an annoyance. But there are practical solutions that might be explored. Why not, for example, see what kind of steering committee they would live with happily and respectfully? NIH might be surprised. Or hammer out some rules that ensure that findings of misconduct reach the public eye in a seemly fashion?

The case for Feder and Stewart and the continuation of their work is that there is a great deal to be learned about the literature by the detailed study of particular aberrations, not only where people have misled others but where they have been misled themselves. The frailties of the literature are mostly accidental, not the consequences of fraud, but they need to be better understood. Feder and Stewart have made a substantial contribution already to the understanding of what makes a reliable text. It will be a misfortune if they cannot continue.

John Maddox

Transgenic plants on trial

Peter Kareiva

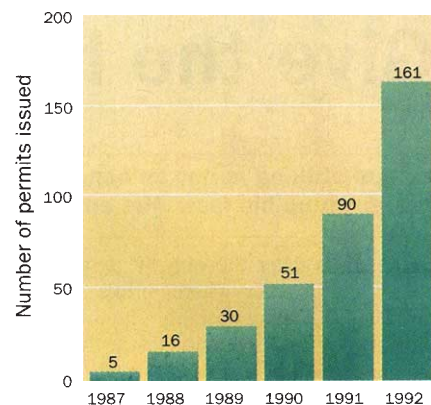
READERS curious to see what a landmark paper looks like should turn to page 620 of this issue¹. There Crawley and colleagues describe an ecological study which will bring one of the hottest debates about the use of genetically engineered plants in agriculture into the realm of rational discourse. The debate centres on the possible invasiveness of such plants into places where they are not wanted. What Crawley *et al.* do is show just how to go about the business of assessing the risks concerned.

Since the US Department of Agriculture started regulating field trials involving transgenic plants, more than 370 permits in 35 states have been issued² (see figure). Genes for a wide variety of agriculturally valuable traits have been inserted into over a dozen crops, as listed in the table, and many of the resultant

cultivars have performed well enough to send companies back to the USDA seeking deregulation of these recombinant genotypes. Deregulation results in a recombinant variety being treated the same as a conventional crop and is, of course, a prerequisite for commercialization of transgenic crops. But environmentalists want to be assured that the ecological risks are minimal. One of those risks is of a new crop escaping from cultivation and invading natural vegetation, a possibility which has been discussed largely in terms of anecdote and platitude based on ideology. Crawley and his team at Silwood Park have finally added what has been glaringly missing from these discussions — a quantitative experimental study of invasiveness in a transgenic plant, the plant concerned being oilseed rape.

To appreciate the value of this new contribution, one needs to recall the disputes that have haunted conferences dealing with the release of genetically engineered organisms. On one side are environmentalists reminding us of the many exotic species that have become pests throughout the world, and pointing out that a genetically engineered organism is in a sense a new type of 'exotic' — a type that may combine traits in ways that could create new pests. On the other side are agronomists boasting of a long history of modifying plants by classical breeding with no ecological disasters on their record.

Both sides are somewhat disingenuous in their arguments. Horror stories about exotic species are not a fair analogy for single-gene modifications in crops that have been domesticated for hundreds or even thousands of years; conversely, the 'good record' of classical breeding is no guarantee of what will happen to plants with traits that go beyond those that have previously been available, especially if the cultivar — sunflower, strawberries, mustards, bermuda grass, for instance — starts with feral tendencies. The battle lines are all the less clear cut because we simply do not know for sure



Number of permits issued per year for field trials involving genetically engineered plants in the United States and Puerto Rico. Because many of the permits cover a single recombinant variety being planted at several sites, the 353 permit-based trials that had been conducted by the end of 1992 actually represent more than 700 sites. (Data from the USDA APHIS BBEP Biotechnology Permits Unit.)

Major cultivars that have been modified using recombinant DNA technology, and the agronomically valuable traits that have been inserted into them

Alfalfa	Herbicide tolerance, virus resistance
Apple	Insect resistance
Oilseed rape (canola)	Herbicide tolerance, insect resistance, modification of seed oils
Cantaloupe	Virus resistance
Corn	Herbicide tolerance, insect resistance, virus resistance, wheat germ agglutinin
Cotton	Herbicide tolerance, insect resistance
Cucumber	Virus resistance
Melon	Virus resistance
Papaya	Virus resistance
Potato	Herbicide tolerance, virus resistance, insect resistance, starch increase, and modification to make a variety of non-potato products such as chicken lysozyme
Rice	Insect resistance, modified seed protein storage
Soybean	Herbicide tolerance, modified seed protein storage
Squash	Virus resistance
Strawberry	Insect resistance
Sunflower	Modified seed protein storage
Tobacco	Herbicide tolerance, insect resistance, virus resistance
Tomato	Virus resistance, herbicide tolerance, insect resistance, modified ripening, thermal hysteresis
Walnut	Insect resistance

This list is abstracted from ref. 2, and includes only traits that have been field tested and not traits such as markers that are often placed in crops in the early stages of product development. When a cultivar has several traits next to it, it does not mean that that cultivar has been modified to include all those traits simultaneously. Traits referred to as insect or virus resistance and herbicide tolerance, are often quite specific and do not apply to all insects, all viruses or all herbicides (details are available from USDA).

what traits will dramatically enhance invasiveness³, and because of the observation that, in fact, some non-transgenic cultivars have become pests⁴ (which makes one wonder whether crop varieties obtained from conventional breeding programmes ought to be of regulatory concern). No amount of barneying will reconcile these different concerns.

Onto this scene enter Crawley and a group of collaborators that includes mathematical ecologists, population biologists and community ecologists, supported by a consortium of industrial and UK government agencies. The team designed an ambitious field experiment in which the population growth of normal oilseed rape plants and genetically engineered genotypes were contrasted across a wide range of environments. 'Invasiveness' was precisely defined as the rate of population increase for oilseed rape from one year to the next, using a simple difference equation model.

The experiment itself is one of the most comprehensive population studies ever undertaken in plant ecology — it involved use of three climatically distinct sites and four habitats (wet versus dry, and sunny versus shady) in each site, making a total of 12 different environments. In each of these environments, various experimental treatments were established: presence or absence of vertebrate grazers, presence or absence of insect herbivores, presence or absence of fungal pathogens, and cultivated or uncultivated background vegetation. Invasiveness was assessed by contrasting population growth for untransformed oilseed rape, for the identical oilseed rape cultivar transformed with a kanamycin marker, and for oilseed rape transformed with both a kanamycin marker and resistance to the herbicide Basta.

The results are strikingly clear: under no environmental or experimental conditions did the transgenic cultivars exhibit different rates of population growth to those of their unmodified counterparts.

Too much should not be read into these results, however: by no means do they provide definitive answers for genetically engineered crops in general. First, history tells us that an ultimately successful invader might initially fail miserably, or barely persist for decades, before exhibiting explosive population growth⁵. Second, there are risks other than invasiveness to be considered — for example the escape of genes through pollen and hybridization could enhance the vigour of existing weeds. Another risk concerns the subtler ecosystem-level effects of widespread transgenic crops, which could come about because of degradation by-products in the soil or associated agricultural practices (such as increased herbicide usage). Finally, the finding of low invasiveness is less likely to apply to transgenic crops with traits that could confer advantages outside cultivation, such as stress tolerance or insect resistance.

The importance of the Silwood study comes not so much from its results, but from its scope and timeliness. In the United States, the USDA has just published its new guidelines for applications for transgenic crop deregulation⁶. These guidelines require firm evidence that the phenotype of the transgenic crop poses no greater risk than does the unmodified plant from which it was derived. Designing practical and consistent protocols to obtain data pertinent to such regulations, on a timescale commensurate with advances in biotechnology, is no small challenge — certainly we cannot expect to apply the Silwood design to every transgenic candidate for deregulation, but it does show the way.

In this context, it is a pity that opportunities to obtain appropriate data have been missed in the hundreds of completed field trials, which have emphasized agronomic performance and have been managed in a way that discouraged multi-generation observations on transgenic populations. So although more than 300 field trials have been carried out and no evidence of 'weediness' has yet emerged, that should not be interpreted as an especially comforting observation — we have been so thorough in containing or destroying all material in field trials that we

could hardly expect to see any hint of problems from these studies. The real question is what will happen when transgenic seeds are widely broadcast year after year in many different habitats, as would be the case if genetically engineered crops are planted commercially.

The Silwood project is indicative of the increasing role of ecology in addressing environmental issues. Academic ecologists are renowned for arguing amongst themselves about all the things they do not know. But when it comes to designing an experiment to measure invasiveness or community impacts, or quantifying the likelihood of gene escape, population

biologists and ecologists do know what to do. Moreover, ecology has been much more than a handmaiden to applied science in this experiment — the study is the largest demographic field experiment ever reported for a plant, and it tells us a great deal about the interplay of disturbance and natural enemies in dictating plant population growth. As ecologists seek answers to practical problems, our understanding of ecological processes is sure to improve. □

Peter Kareiva is in the Department of Zoology, University of Washington, Seattle, Washington 98195, USA.

INSECT VISION

Old twist in a new tale

Michael F. Land

MOST of the photoreceptors of honey bees are constructed like a corkscrew, with a helical twist. Why should that be so? Rüdiger Wehner and Gary Bernard have looked into the matter, and in a paper in *Proceedings of the National Academy of Sciences*¹ describe how they have come up with the answer. It centres on the conflicting demands of identifying polarized light and colours, and very neat and satisfying it is too.

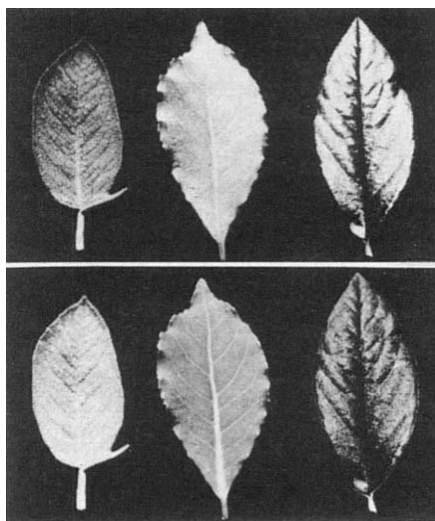
The photoreceptors of most invertebrates have a polarization analysing capability built into their geometry. The finger-like microvilli containing the photopigment have more molecules oriented parallel to their long axes than across them, and because of this the receptors respond best to light polarized in a plane that contains the microvillar

axes. This property is often valuable, and is exploited by many insects and other arthropods as a navigation aid, the polarization field of the sky providing a valuable alternative to the Sun as a temporary compass. In other instances, for example in the aquatic bug *Notonecta*, the analyser capability is used to detect water surfaces, as these polarize light — this is why polaroid sunglasses can remove the reflected glare.

Wehner and Bernard conclude that there are some circumstances where this capability is actually a nuisance and has to be dispensed with, because it gets in the way of other visual functions such as colour vision. Therein lies their solution of the old mystery of why the photoreceptors of bees are twisted. Without the twist, argue the authors, polarization would make the bees see the wrong colours in plants.

Their case is based on two aspects of the way light is polarized by natural objects. First, all non-metallic reflecting surfaces, water being one example, polarize light strongly at certain angles of incidence. Second, leaves and petals vary in their specular reflectance, depending on how waxy they are — for example, sage leaves are matt and bay leaves are shiny (see figure). So the degree and direction of polarization reflected from vegetation will vary greatly.

This is no problem for us as we are polarization-blind. But for a bee it will cause difficulties, because the photopigments responsible for its colour vision (green-, blue- and ultraviolet-absorbing) are contained in receptors with different microvillar orientations². Thus, potentially, each receptor will give a signal that depends not only on intensity and wavelength but also on the direction and degree of polarization. Wehner and Bernard describe the consequences dramati-



Leaves (from the left) of sage, bay and cotoneaster photographed in light polarized parallel to the leaf surface (top) and at right angles (below). Notice that the relative brightnesses of the matt and shiny leaves reverse.

1. Crawley, M. J., Hails, R. S., Rees, M., Kohn, D. & Buxton, J. *Nature* **363**, 620–623 (1993).
2. United States Department of Agriculture, APHIS Biotechnology Permits Unit, database release 12 May 1993.
3. Williamson, M. *Experientia* (in the press).
4. Ellstrand, N. & Hoffman, C. *Bioscience* **40**, 438–442 (1990).
5. Drake, J. H. et al. *Biological Invasions: A Global Perspective* (Wiley, New York, 1988).
6. United States Federal Register, Part X, Department of Agriculture, 7 CFR Part 340, Final Rule, March 31 1993.

cally: "... when zig-zagging over a meadow with all its differently inclined surfaces of leaves, the bee would experience pointillistic fireworks of false colors that would make it difficult to impossible to detect the real colors of the flowers". They demonstrate the reality of this surmise by computing the havoc caused in the bee's colour triangle when it views reflections from a dandelion leaf at different angles.

The resolution of this unhappy state of affairs is the twist in the receptors. First demonstrated in 1975³, and subject to much dispute since, it is now well established that the long receptors twist at the rate of about 1° per micrometre. This prevents the microvilli from staying parallel throughout the receptor's length, and so abolishes its ability to respond selectively to the plane of polarization. And so, of course, this allows each type of receptor to take part unambiguously in the bees' trichromatic colour vision system.

The story has added spice because, many years ago, it was in honey bees that von Frisch demonstrated polarized light vision; he showed that bees respond to sky polarization, which they use for navigation⁴. The receptors concerned are not distributed throughout the eye, however, but are located in a special upward-pointing 'dorsal rim' area of the eye. Here, uniquely, the receptors are not twisted, so that their polarization sensitivity is preserved⁵. One has to be impressed by the simplicity and versatility of nature's optical engineering. □

Michael F. Land is in the School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK.

1. Wehner, R. & Bernard, G. D. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4132–4135 (1993).
2. Gribakin, F. G. *Nature* **233**, 639–641 (1969).
3. Wehner, R., Bernard, G. D. & Geiger, E. *J. comp. Physiol.* **A104**, 225–245 (1975).
4. von Frisch, K. *Tanzsprache und Orientierung der Bienen* (Springer, Heidelberg, 1965).
5. Labhart, T. *J. comp. Physiol.* **A141**, 19–30 (1980).

ASTRONOMY

Light on the faint Universe

M. G. Edmunds

COSMOLOGY has so few well-established facts that we must welcome any attempt to broaden its empirical basis. A recent meeting* tried to fit the numbers and properties of galaxies — the basic observed building blocks — into the larger picture of the evolution of the Universe.

Checks can be made to see whether the theory of galactic evolution violates other constraints — such as the background radiation observed from the sky at various wavelengths. This background includes both the cosmic microwave background radiation, which originated long before the galaxies formed, and the total of all other radiation emitted by stars or other sources. It is also important to identify all the remaining normal baryonic (nuclear) matter — within or outside galaxies — which we have not yet recognized, but whose mass is predicted from the observed abundances of elements formed in the Big Bang.

Background

S. Bowyer (Univ. California, Berkeley) showed just how difficult it has become to detect a significant ultraviolet and optical background originating from outside our own Galaxy. Fixing the level of the background would allow some estimate of the total amount of baryonic mass in the Universe that has formed into radiating stars. The main problem is the effect of

'cirrus' dust clouds in our Galaxy — originally found by the infrared satellite IRAS — which scatter ultraviolet and optical light and make it very hard to separate out the true extragalactic component. R. S. Ellis (Univ. Cambridge) reported that at least some significant extragalactic component is implied by recent balloon observations at a wavelength of 200 nm by astronomers at Marseille of a region of sky that has been well studied at optical wavelengths. Extrapolation of the relative numbers of sources observed in the ultraviolet suggests that the background should lie towards the top of the range suspected from general background measurements. But the smallness of spatial fluctuations in the optical background led K. Mattila (Univ. Helsinki) to suspect that the sources of the background luminosity must partly be spread between the galaxies, smoothing out the distribution.

A background at rather shorter wavelengths (XUV) has considerable implications for galaxy formation. This radiation could inhibit cooling of protogalactic clouds, and J. Sommer-Larsen (Niels Bohr Institute) found that including it in models of galaxy formation gives galaxies that are far more realistic than when it is neglected. Such radiation would efficiently ionize the intergalactic medium, and a search for ionized helium lines (He II), rather than neutral hydrogen (H I), in the absorption ultraviolet spectra of quasars might have been the technique of choice for seeking intergalactic matter.

Unfortunately, an extensive feasibility study (P. Jakobsen, European Space Agency) suggests that not enough ultraviolet flux survives from quasars to make observations possible in the foreseeable future — a view that is rather more pessimistic than has recently been put forward in the literature¹.

If, as expected, the principal sources of the background radiation are stars in galaxies, then these stars must also have produced heavy elements. B. Pagel (NORDITA, Copenhagen) showed that there are no really outstanding contradictions between background levels and observed heavy-element abundances.

Excess

An obvious test to check if we are missing any major sources of light (and hence 'hidden' baryonic matter) is to see if the total luminosity extrapolated from counts of galaxies is consistent with the backgrounds. Again, there seems to be no major conflict. But interpreting the number counts is a rather messy game. Nonetheless, both Ellis and L. Cowie (Univ. Hawaii) agreed that there appear to have been more galaxies around at times corresponding to redshifts of 0.5–1 than there are today. (Increasing redshift corresponds to observations closer to the Big Bang; the most distant quasars known are just short of redshift equal to 5.) On the other hand, Ellis suggested that there is good evidence² that the population of large, bright elliptical galaxies has remained unchanged since redshift 1, except for some fairly quiescent evolution of the constituent stars. There is evidence, from the uniformity of their colours, that their stars formed largely at about the same time, corresponding to redshifts 2–5.

This undramatic picture of evolution is reinforced by observation of magnesium absorption lines in quasar spectra. The absorption probably arises in the disks of bright galaxies, and again there is evidence that these galaxies have not changed much since a redshift of about 1. So what are the extra galaxies seen at these redshifts? Inevitably one is driven towards a picture in which galaxies (other than perhaps the most luminous) are formed by merger of a number of smaller fragments, perhaps four to six, between redshift 1 and now. The active star formation triggered by the mergers could explain the appearance of a population of blue galaxies seen at a redshift of 0.5, which seem since to have faded or to have hidden themselves. Unified models of the photometric evolution of galaxies involving merging were presented at the meeting (B. Rocca-Volmerange, Univ. Paris; G. Kauffmann, Univ. Cambridge). And merging models are becoming quite popular in the literature^{3,4}.

There are theoretical arguments that the merger rate should have peaked some-

* Nordic Workshop Symposium, *Dim Galaxies, Background Radiation and Baryonic Dark Matter*, Copenhagen, 3–7 May 1993.

time in the past, but the apparent lack of strong clustering of the faint blue galaxies⁵ — which might be expected if they are involved in merging systems — remains a problem. Ellis showed some direct images of these blue galaxies taken with the Canada–France–Hawaii telescope under very good seeing conditions, but first impressions are that they are an odd assortment of single and multiple objects.

There is still hope that there may be large amounts of yet-to-be detected normal baryonic matter in the form of stars and gas in very low surface brightness galaxies. The mass involved could be up to a half of that seen in the more easily observed bright galaxies. S. McGaugh (Univ. Cambridge) emphasized that it is already clear that old assumptions about the surface brightness of disk galaxies give estimates of number densities of dimmer objects too low by orders of magnitude. The dim objects are typically irregular galaxies or very late-type spirals (which lack an obvious central bulge) (Th. van der Hulst, Univ. Groningen). In clusters they are much more frequent relative to

brighter galaxies than in the general field (M. Disney, Univ. Wales, Cardiff). Only a few (N. Bergvall, Univ. Uppsala) show signs of being genuinely ‘young’ galaxies, and it seems more likely that they have just evolved very slowly. There has been little success in the hunt for the objects which are the large, luminous galaxies in their formation stages. Cowie has found that in deep infrared images of the sky, one in five of the faintest objects are very red, and these could be very high redshift galaxies in formation. Studying them will be a great challenge to the new generation of 8-metre optical/infrared telescopes. □

M. G. Edmunds is in the Department of Physics and Astronomy, University of Wales, College of Cardiff, PO Box 913, Cardiff CF2 3YB, UK.

1. Miralda-Escudé, J. *Mon. Not. R. astr. Soc.* **262**, 273–276 (1993).
2. Aragón-Salamanca, A., Ellis, R. S., Couch, W. J. & Carter, D. *Mon. Not. R. astr. Soc.* **262**, 784–794 (1993).
3. Lacey, C. & Cole, S. *Mon. Not. R. astr. Soc.* **262**, 627 (1993).
4. Treyer, M.-A. & Silk, J. *Astrophys. J.* **408**, L1–L4 (1993).
5. Efsthathiou, G. *et al.* *Astrophys. J.* **380**, L47–L50 (1991).

NEUROPSYCHOLOGY

The scratchpad of the mind

Marcus E. Raichle

ALL of us have had the experience of knowing a telephone number while we are placing a call or the name of an individual to whom we have just been introduced. But moments later, sometimes to our frustration and embarrassment, recall is impossible. Psychologists suggest we possess a working memory store¹ to hold important information in mind for brief periods of time. Storage is temporary and the capacity is strictly limited². The mystery is how such an essential faculty of the mind is implemented in the brain. Two reports^{3,4}, in *Nature*, one on page 623 of this issue³, now present fresh insights into this mystery. Both of the groups concerned used positron emission tomography (PET; see ref. 5 for review) to study brain functional anatomy during working memory tasks. Changes in blood flow, measured by PET, provided maps of the brain areas involved.

Working memory is distinct from our ability to store and recall the innumerable facts and events that accumulate over a lifetime⁶ (variably referred to as explicit recall or declarative memory). Amnesic patients may have profound deficits in declarative memory, but they can remember a short list of items for several minutes if they rehearse it and are not distracted. The brain areas essential for declarative memory, such as the hippocampal formation, are well known⁶; those involved in the working memory system are not. With

rare exceptions⁷, strokes and other localized injuries to the brain do not impair working memory in any predictable manner whereas bilateral damage to the hippocampus always produces complete anterograde amnesia⁸.

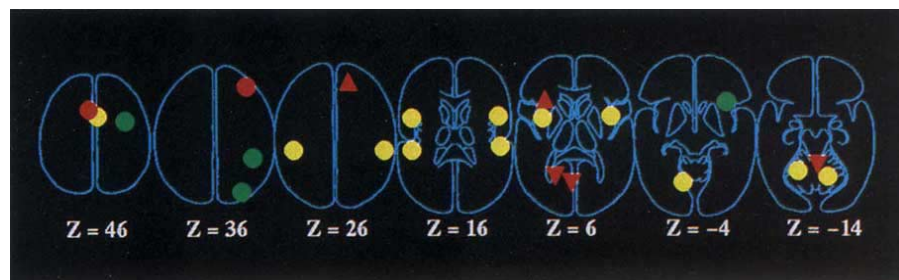
Baddeley¹ has proposed that working memory consists of a central controller or executive and a group of slave systems. The central executive is responsible for the allocation and coordination of the brain's attentional resources and information from the slave systems. The two most frequently proposed slave systems are one for inner speech, which allows silent

rehearsal of online information, and a visuospatial sketch pad, responsible for maintaining visuospatial information. The two new brain-imaging studies^{3,4} give intriguing glimpses of the brain anatomy underlying these systems.

Jonides and his colleagues³ asked subjects to remember the spatial location of groups of dots briefly presented on a television monitor. During the PET scans the subjects were tested on their ability to remember the location of each group of dots. Four widely separated areas of the brain, all on the right side, were activated by this task. These included the right frontal, right premotor, right parietal and right occipital (visual) cortex. So a task requiring the brief storage of visuospatial information required a widely distributed network of brain areas, one including areas of visual cortex but extending well outside the visual system itself. Furthermore, these areas were concentrated on the right side of the brain emphasizing, once again, the lateralization of function which is so characteristic of the human brain.

In the study by Paulesu, Frith and Frackowiak⁴, subjects had to remember six visually presented letters of the English alphabet. The subjects were encouraged to rehearse the letters silently while awaiting the appearance of a probe letter on the television monitor. Their task was to determine whether the probe letter matched any of the six letters they rehearsed in working memory. Entirely different areas of the brain were activated compared to those observed to be involved in the visuospatial working memory task³. There were also changes on the left side of the brain as well as the right. This left-sided involvement is consistent with the lexical nature of the task.

A preliminary study in which I have been involved⁹ provides further information. Here subjects were presented visually with five pseudo words (such as ‘floop’) or five words in working memory. Nothing was required of them during the



The location of brain responses while briefly remembering a spatial location³ (green), letters of the English alphabet⁴ (yellow) and words⁹ (red). In the study of words⁹, subjects were divided into those performing well (upright triangles) and those performing poorly (inverted triangles). Areas common to both groups are shown as red circles. These locations are plotted on horizontal sections of the human brain beginning at the top of the brain (left). The front of the brain is located at 12 o'clock and the left side is to the reader's left. The brain sections are drawn from the stereotaxic atlas of Talairach and Tournoux¹². The numbers under each brain section refer to the millimetre distance of the plane of section above (positive) and below (negative) a horizontal plane passing through the anterior and posterior commissures of the brain¹².

PET scan other than to remember the words or pseudo words. The subjects were given no instructions about strategy, only that they would be asked to recall the words or pseudo words after the PET scan. Subjects were divided into good and bad performers according to their recall of five pseudo words. In each group both right prefrontal cortex and the supplementary motor cortex were activated. But the two groups differed in that the good performers used a left premotor and an anterior cingulate area whereas the bad performers used areas of the left visual cortex and cerebellum, suggesting there was a visual (bad performers) against a phonological (good performers) difference in strategy.

Where does all this leave us? First, it seems that working memory is a distributed process with many, widely separated areas of the brain acting in support of a particular task. Task demands and individual strategy differences dramatically alter the anatomy of these brain circuits. All these features explain why working memory has been difficult to study with brain-damaged patients, and the challenge is to unravel the unique operations contributed by each brain area within a circuit. Second, why do individuals select a particular strategy, sometimes to the detriment of their performance⁹? Do training and experience matter? If so, how might we wish to intervene? Third, active brain areas are frequently lateralized in human working memory tasks in contrast to the areas reported to be involved in monkeys¹⁰, meaning that caution must be exercised in making direct comparisons.

Finally, how do we distinguish the central executive from supporting slave systems? Indeed, is such a distinction meaningful? Maybe Sir Charles Sherrington was right when he wrote¹¹, "Pure conjunction in time without necessarily cerebral conjunction in space lies at the root of the solution of the problem of the unity of mind". □

Marcus E. Raichle is in the Mallinckrodt Institute of Radiology, Washington University School of Medicine, 510 South Kingshighway, St Louis, Missouri 63110, USA.

1. Baddeley, A. *Science* **255**, 556–559 (1992).
2. Miller, G. *Psychol. Rev.* **63**, 81–97 (1956).
3. Jonides, J. *et al.* *Nature* **363**, 623–625 (1993).
4. Paulesu, E., Frith, C. D. & Frackowiak, R. S. J. *Nature* **362**, 342–345 (1993).
5. Raichle, M. E. *Curr. Neurol.* **9**, 161–178 (1989).
6. Squire, L. R. *Psychol. Rev.* **99**, 195–231 (1992).
7. Warrington, E. K. & Shallice, T. *Brain* **92**, 885 (1969).
8. Scoville, W. B. & Milner, B. J. *Neurol. Neurosurg. Psych.* **20**, 11–21 (1957).
9. Raife, E. A., Fiez, J. A., Raichle, M. E., Balota, D. A. & Petersen, S. E. *Neurosci. Abst.* **18**, 932 (1992).
10. Goldman-Rakic, P. S., Funahashi, S. & Bruce, C. J. in *Cold Spring Harb. Symp. quant. Biol.* Vol. LV 1025–1038 (Cold Spring Harbor Laboratory Press, 1990).
11. Sherrington, C. *The Integrative Action of the Nervous System* 2nd Edn, 381 (Yale University Press, New Haven, 1947).
12. Talairach, J. & Tournoux, P. *Co-Planar Stereotaxic Atlas of the Human Brain* (Thieme, New York, 1988).

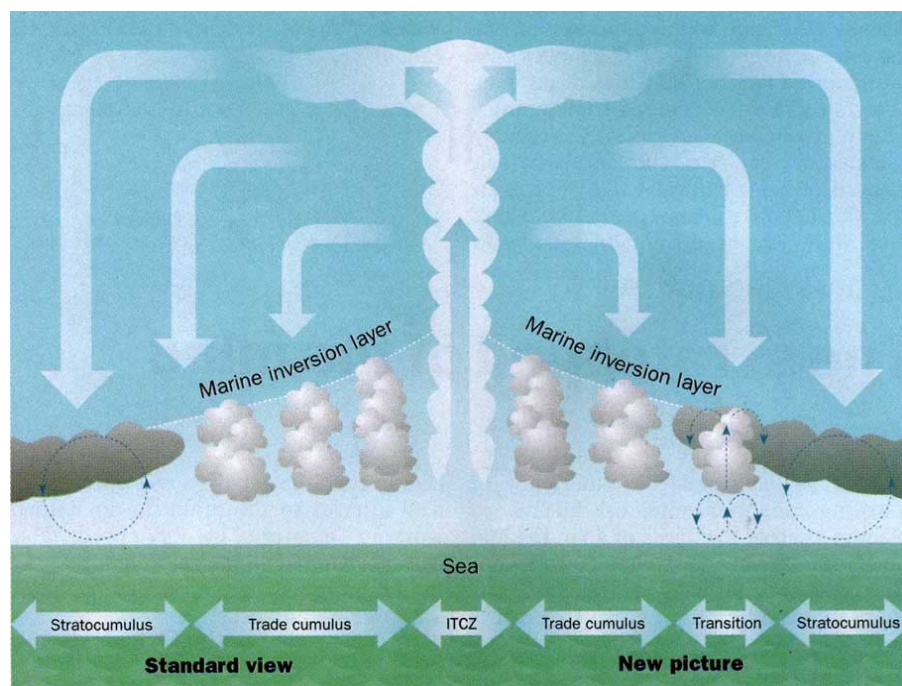
Breaking up is hard to do

Gabrielle Walker

THE mechanisms by which the extensive decks of grey, stratocumulus clouds over the subtropical oceans break up to form individual, cumulus clouds have long taxed atmospheric scientists. Stratocumulus clouds are a key component in the climate, cooling the Earth by reflecting solar radiation back into space. Their behaviour must be understood if we are to predict their role in a changed climate with any accuracy. A battery of observations from land, air and sea has now thrown new

sion layer forms with warm, dry air forced to lie above the cooler, moister air close to the sea surface.

It is in this layer that the cumulus and stratocumulus clouds form. The grey, drizzly stratocumulus decks develop at subtropical latitudes where the inversion layer is at its lowest. Closer to the Equator, the inversion layer is higher and weaker, and the white fair-weather trade-cumulus clouds predominate. It seems likely that when the inversion layer



From the subtropics to intertropical convergence zone (ITCZ) near the Equator, stratocumulus and trade cumulus clouds form below the marine temperature inversion layer, where warm, downwelling air meets cool, moist marine air. The change from one cloud form to the other was thought to be sharp (left), but the ASTEX results reveal a transition region (right). Changes in circulation below the cloud top could account for this: with two distinct vertical cells, air laden with water vapour from the sea surface cannot feed the stratocumulus deck so readily.

light on the mystery, revealing an unexpectedly complicated structure beneath the low-lying cloud deck. The observations, from the Atlantic Stratocumulus Transition Experiment (ASTEX), were reported last month at a meeting in Baltimore*.

Close to the Equator, the trade-wind systems of the two hemispheres converge at the intertropical convergence zone (ITCZ). Air rises here in a narrow ascending branch of the 'Hadley' circulation cell (see figure), and towering cumulonimbus clouds form, with vast, spreading anvil heads. The air moves polewards at high altitudes and then sinks, warming as it is compressed so that a temperature inver-

reaches a certain height there is no longer enough water supplied from the sea surface to balance the entrainment of dry air from above the cloud (which may also increase towards the ITCZ), and the stratocumulus deck breaks up to form the trade cumulus. According to the standard model, there is a sharp transition between these two cloud types. But the ASTEX results tell a different story.

The ASTEX investigators, working off the Azores last summer, discovered a transition region in which cumulus clouds appeared to be forming beneath the stratocumulus deck, sometimes penetrating the top of the deck and spreading out like a miniature anvil head. According to participants at the conference, it is not yet clear how the cumulus clouds might influ-

* American Geophysical Union Spring Meeting, Baltimore, 24–28 May 1993.

ence the break-up of the stratus deck, but they seem likely to have a significant role. The cumulus clouds appeared to be conveying new aerosol particles (some of them cloud-condensation nuclei) of varying sizes into the stratus deck, considerably changing its radiative properties. Such particles might stabilize the cloud deck, increasing its lifetime. Alternatively, they might intensify the drizzle from the cloud, reducing its water load and so increasing its chances of breaking up. The cumulus clouds that penetrate the cloud top could increase entrainment of dry air from above the clouds, also increasing the likelihood of break-up.

Various theories already exist that may explain the formation of the cumulus/stratocumulus melange. One possibility is solar heating within the cloud. In the stratocumulus regions where the marine inversion layer is relatively low, longwave radiative cooling of the cloud top results in large-scale eddies beneath, which fill the volume between the cloud top and the sea surface. But during the day, absorption of solar shortwave radiation heats the cloud, and this splits the eddies into smaller cells (see figure). This decouples the cloud from its supply of moisture at the sea surface, and can break up the stratocumulus deck.

At the same time, the decoupling moistens the air near the sea surface, leading to the extra cumulus clouds below the deck. Alternatively, drizzle from the cloud might be responsible for the decoupling of the stratocumulus layer and the region below; evaporation of the drizzle before it reaches the sea surface takes latent heat from the atmosphere, cooling the air beneath the cloud to give a local density inversion layer. This could then be the base for the cumulus formation.

It is now down to the modellers to try to distinguish between these mechanisms, and determine how to parametrize the processes in three-dimensional climate models. Meanwhile, a stroke of good fortune enabled the ASTEX investigators to study the effects of industrial aerosol pollution on the cloud formation. While they were performing their measurements, a patch of extremely dirty continental air arrived overhead from Europe. The clean and dirty air patches had markedly different cloud characteristics — all the more striking because the patches were associated with similar sea-surface temperatures and were downwelling with about the same strength.

Not surprisingly, the cloud droplets in the dirty air were smaller and more numerous than those in the clean air, because the same amount of water was shared by more condensation nuclei. This well-known effect means that clouds associated with dirty air reflect more sunlight, providing a potential counterbalance to the warming caused by in-

creased atmospheric concentrations of greenhouse gases.

But the ASTEX investigators also found that the complex, stratocumulus/cumulus melange did not appear to form in the dirty air. This could be because the smaller cloud droplets are less prone to drizzle and also absorb less shortwave radiation, making each of the decoupling mechanisms less likely to occur. The

break-up of the stratocumulus deck might therefore be significantly affected by the influx of industrial aerosols, opening up a new route by which the aerosols produced by fossil fuel burning may affect cloud radiative properties, and thereby influence the Earth's climate. □

Gabrielle Walker is an assistant editor of Nature.

OCEAN CHEMISTRY

Ruling in the improbable

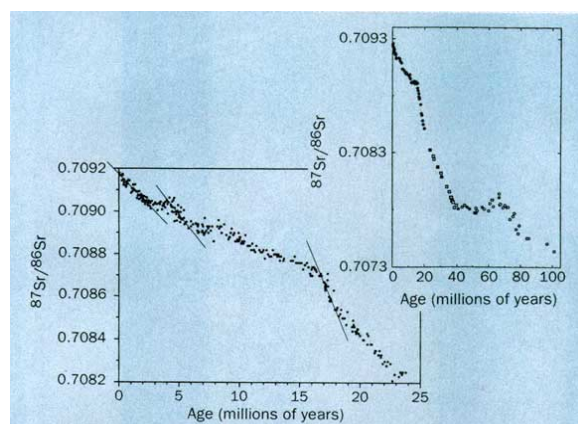
Philip N. Froelich

SALTS in the ocean, the soluble products of continental and seafloor weathering on their way to burial in sediments and eventual subduction, seem to remain in a remarkably steady long-term balance. Over geological time, a few constituents drift slowly in harmony with the changing global forces that create and destroy crust — the great hundred-million-year cycles of seafloor spreading, continental upheaval and erosion that modulate the

dweller's palaeoceanographic tools not only for unravelling the details of past ocean temperature, circulation and nutrient changes, but also for marking the timing of the ice ages. They incorporate the seawater oxygen isotope signals imparted by the waxing and waning of the great continental ice sheets.

The isotope composition of strontium in sea water, however, is another matter. The seawater ratio today (0.709) reflects a

balance between two dominant crustal sources: weathering of ^{87}Sr -rich continents (rivers average 0.712) and ^{87}Sr -poor seafloor basalts (hydrothermal fluids average 0.703). For a steady state, the strontium is then two-thirds riverine and one-third hydrothermal³; combined with the known river strontium flux, this indicates precisely the same hydrothermal strontium flux as that estimated from the convective ^3He and heat fluxes at mid-ocean ridges. An evolving consensus⁴ holds that the long-term increase in the ocean ratio over the past 100 million



Two composite views of the long-term rise in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ (from refs 10, 11). The new record of Clemens *et al.* covers the most recent 500,000 years.

geochemical cycles and the long-term evolution of climate¹. Now, to the astonishment of geologists, ocean chemists and palaeoclimatologists alike, we are told on page 607 of this issue² that one of the key markers of long-term ocean chemical change beats in phase with the 100-thousand-year orbital rhythms that pace the ice ages. Can the oceans change so rapidly? Something is terribly wrong, leaving Earth scientists with an uncomfortable, Sherlock Holmes-like choice between the impossible and the improbable.

Clemens *et al.* provide in this issue a detailed record of changes in the seawater strontium isotope ratio over the past half-million years, as monitored by the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in the calcite shells of planktonic foraminifera that settle to the ocean bottom. The shells of these ocean-surface

years (see figure) must be driven by continental rather than hydrothermal processes, most probably increasing river $^{87}\text{Sr}/^{86}\text{Sr}$ ratios associated with episodic uplift and unroofing of the Himalayas⁵. The continental collision that caused this is responsible for both the modern Indian monsoonal climate and the uniquely ^{87}Sr -rich rocks and rivers (the Ganges and Brahmaputra river ratios exceed 0.720; ref. 6) that are slowly lifting the ocean $^{87}\text{Sr}/^{86}\text{Sr}$, more rapidly so during periods of climate deterioration 20–15, 5.5–4.5 and 2.6–0 million years ago. This climate–tectonic coincidence suggests⁴ a link between Tibetan uplift and global climate associated with more intense chemical weathering and drawdown of CO_2 .

Within this long-term rise in $^{87}\text{Sr}/^{86}\text{Sr}$,

Clemens *et al.* see Milankovitch cycles embedded in the past half-million years. (Milankovitch identified a correlation between climate cycles and the periodic variations in the Earth's orbit about the Sun.) Their $^{87}\text{Sr}/^{86}\text{Sr}$ record is punctuated by deglacial rises and glacial falls that are coherent (the amplitude variations match) and in phase (no leads or lags) with oxygen isotope fluctuations (growth and decay of glacial ice). This implies strong and fast connections between the continental component of climate change and the forces driving the seawater $^{87}\text{Sr}/^{86}\text{Sr}$ changes.

Clemens *et al.* recognize that their data are difficult to reconcile with the present paradigm. First, strontium has a residence time in the sea of several million years, too long to accommodate the apparent rates of these isotope changes. Even worse, the systematics of present-day strontium weathering and river $^{87}\text{Sr}/^{86}\text{Sr}$ ratios⁶, including those draining the Himalayas and ^{87}Sr -rich continental shields (glaciated

during the ice ages), preclude the requisite changes in the river flux or ratio needed to drive the amplitudes of their observed changes. Turning rivers off altogether during glacials hardly seems reasonable.

The second, more substantial stumbling block is that the measured changes in the ocean ratio are minuscule, all contained within their 2σ error limits. The maximum glacial-to-interglacial changes in $^{87}\text{Sr}/^{86}\text{Sr}$ (0.000020, or 20 p.p.m.) are at the edge of the best mass spectrometer reproducibility (8 p.p.m.). Alas, the standard deviation of the entire data set is 8 p.p.m. The reported glacial-to-interglacial differences are thus supportable only in the statistical sense. Spectral comparison of the authors' $^{87}\text{Sr}/^{86}\text{Sr}$ record with the global ocean oxygen isotope record indicates significant covariation for the 100,000 year Milankovitch cycle (less so for the 41,000-year cycle). Their statistical case is robust, based on almost two decades of matching Late Pleistocene cli-

mate records to Earth-orbital rhythms⁷. So what could possibly be wrong?

The simplest answer is to dismiss the spectral analysis as analytically unresolvable, that the devil is in the details of the tiny changes in the measurements. Clemens *et al.* have partially answered this problem by analysing all 77 data points in duplicate or triplicate, and by 'super-cleaning' a subset of their foraminifera and rerunning them. The 17 samples of the subset have no non-calcite contamination phases that might carry non-seawater ratios and offset the measured ratios. But the ghosts of mass spectrometry hide in mysterious places. Those who wish upon fast ocean variations see signal; those who do not, see noise.

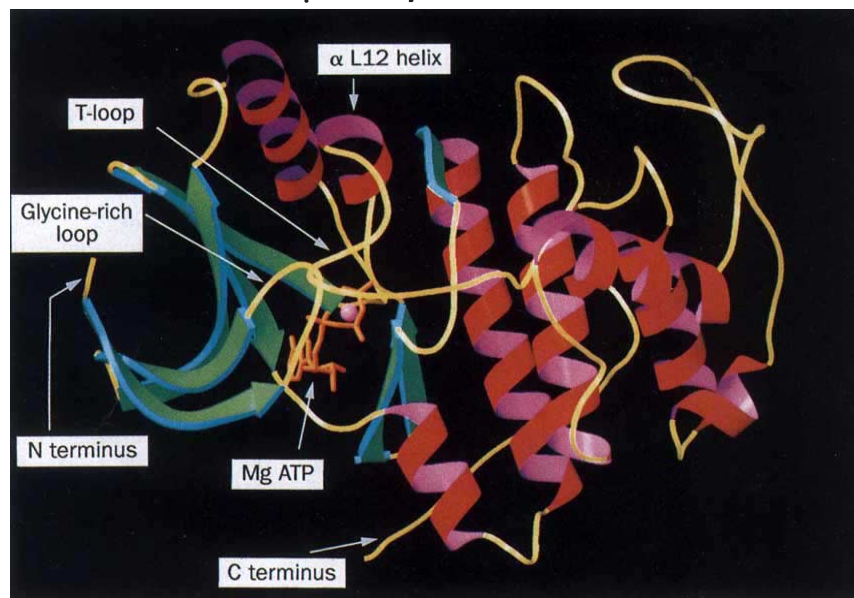
Another possibility is that ocean $^{87}\text{Sr}/^{86}\text{Sr}$ was not homogeneous during glacial periods as it is today. The foraminifera come from an Ocean Drilling Program site in the equatorial Indian Ocean, only 1,900 kilometres south of the mouths of the great Himalayan rivers that could raise the ratio locally but not globally. Climate-induced fluctuations in the Indian monsoon, which modulate rainfall and thus the ^{87}Sr -rich Himalayan river flows, might impart the Milankovitch precessional (23,000-year) cyclicity to their $^{87}\text{Sr}/^{86}\text{Sr}$ signal. Yet the cross-spectral analysis shows no evidence of such forcing, confounding the search for a direct local link to the Himalayas. In addition, an earlier less-detailed $^{87}\text{Sr}/^{86}\text{Sr}$ record from a piston core in the western equatorial Pacific⁸ contains similar changes over the past 250,000 years, so the effect is apparently global. (Although the Cambridge team that produced the record is now itself uncertain about its validity⁹.)

Are the present river $^{87}\text{Sr}/^{86}\text{Sr}$ systematics poor analogues for the glacial past? Were glacial continental drainage patterns and $^{87}\text{Sr}/^{86}\text{Sr}$ ratios so dramatically different that we are missing some subtlety? There might be a case for this view if built on a geochemical proxy other than $^{87}\text{Sr}/^{86}\text{Sr}$, but the well-established ^{87}Rb - ^{87}Sr dating method and the concomitant large data set for $^{87}\text{Sr}/^{86}\text{Sr}$ in rocks and fluids virtually precludes oversight in this instance.

So we are left with one of the choices that Clemens *et al.* mention last — pulsations of the seafloor hydrothermal fluxes with the ice ages. Fluxes would need to relax on deglaciations, driving up seawater $^{87}\text{Sr}/^{86}\text{Sr}$ in steps. What mechanisms might be responsible? Tectonic flexure of the upper mantle, by loading and unloading the enormous masses of water-ice between ocean basins and continents, might be considered, but is too slow to produce phase lock with climate change. Changes in the hydrothermal isotope ratio (0.703) also seem unlikely.

But there is one unexpected possibility. The 130-m rise and fall of sea level (by

The phosphate's tale



THIS illustration is not a pasta cook's nightmare but the structure of cyclin-dependent kinase 2 (CDK2), as reported on page 595 of this issue. Like other protein kinases, CDK2 catalyses the transfer of γ -phosphate from Mg ATP to a protein substrate, this particular enzyme being implicated in control of the G1- and S-phase events of the human cell cycle. It is the catalytic subunit of human CDK2 that is shown here. It consists of two domains: an amino-terminal lobe (mainly green β -sheet, left) and a larger carboxy-terminal lobe (mainly red α -helix, right). The Mg ATP sits at the bottom of the substrate-binding cleft between the two lobes. Unlike other protein kinases, where the catalytic subunit is fully active, the CDK2 apoenzyme is unable to phosphorylate its substrate. It seems that the glycine-rich loop, and indirectly the α L12 helix, force the Mg ATP into an unfavourable conformation for γ -phosphate transfer; also, a large loop — the T-loop — blocks the substrate-binding cleft. Yet for the cell cycle to proceed CDK2 must be activated, albeit transiently. The initial step is thought to involve the binding of cyclin A to the enzyme's amino-terminal lobe. This may destabilize the α L12 helix, allowing the Mg ATP to assume a conformation for phosphate transfer and loosening up the T-loop. Full activation of CDK2 requires the phosphorylation (by another kinase) of threonine 160, at the tip of the T-loop. This phosphate could pin the loop back against the carboxy-terminal lobe, through ionic interactions, allowing the substrate access to the active site.

G. R.

definition, coherent with the oxygen isotope changes) must raise and lower the depth in the crust at which the hydrothermal reactions occur. The reaction of basalt with sea water is poised at water's thermodynamic critical point, at a temperature of 400 °C and pressure corresponding to a depth of 5,000 m below the sea surface. This places the reaction zone kilometres deep within the mid-ocean-ridge crest. Here convecting sea water approaches the cracking front of recently molten basalt. This critical point in space, temperature and pressure represents one of the great thermodynamic balances of the Earth — the intersection of the Earth's internal heat gradient with the standing height of cold water circulating through the Earth's crust. Could sea-level changes cause fluctuations in high-temperature basalt alteration and hydrothermal strontium fluxes, in phase with climate? Do hydrothermal fluxes turn off during deglacial rises in sea level?

Regardless of the mechanisms that might be causing these rapid $^{87}\text{Sr}/^{86}\text{Sr}$ changes (if they are real), one concern emerges: the $^{87}\text{Sr}/^{86}\text{Sr}$ ratio in sea water may not be at steady state on glacial-interglacial timescales. Estimates of hydrothermal fluxes based on strontium isotopes are not assured until the nature of these variations can be fathomed (or discounted) and their time transients refined sufficiently to provide an assessment of the forces driving them. As Edmond⁶ stated: "The ultimate utility of environmental paleorecords is that they constrain models of processes of climate and geological change". Once again, strontium isotopes may provide us with clues to the links between tectonics and climate on a still-evolving Earth.

The effort continues to confirm or refute the Clemens' record and to extend it backwards in time. Also, the search is on to find other oceanic clues of rapid continental or hydrothermal variations. Osmium and lithium isotopes might fit the bill, but development of these traces is in its infancy. Unlike Sherlock Holmes, earth scientists are unhappy with the improbable, even when they have eliminated the impossible. □

Philip N. Froelich is at the Lamont-Doherty Earth Observatory, Columbia University, Palisades, New York, USA.

Four legs good, two legs better

Bernard Wood

BIPEDALISM is a fundamental human characteristic yet virtually nothing is known about its origins. Two very different sets of studies — one (physiological) by P. E. Wheeler¹, the other (behavioural) by K. D. Hunt² — now threaten to put our understanding of why we stand and walk on two legs on a firm footing.

It is not difficult to discern why only modest progress has been made towards

locomotion, or the other way about? To what extent was climbing preadaptive for bipedalism and, if so, would a patchy fossil record be able to discriminate between the behaviours? Was bipedalism an 'all-or-nothing' adaption, or was it a variable component in a locomotor repertoire? If so, what factors would cause early hominids and their precursors to increase their emphasis on bipedal posture, or locomotion, or both?

The fossil record provides some clues. The preserved anatomy of the earliest hominid species, *Australopithecus afarensis*, and the footprints at Laetoli, a rare and precious example of 'fossilized behaviour' are clearly relevant. This evidence shows that by 3.6 million years ago an early hominid species was capable of bipedal locomotion. But its skeleton is a perplexing palimpsest of an ape-like upper body combined with hips, legs and feet showing a mixture of features, some similar to those of modern humans, some ape-like and some frankly unique.

Wheeler and Hunt adopted contrasting strategies to investigate human bipedalism and its origins. Wheeler's latest paper¹ is one in a long series in which



Stepping out — but chimpanzees are not whole-hearted locomotor bipeds.

he takes a 'bottom-up' modelling approach. His first principles are physiological ones, and he asks in what circumstances bipedal posture and locomotion would have enabled early hominids to optimize their energy resources and reduce both heat stress and their requirement for water.

The results stem from experiments and calculations based on a 1:5 scale model of a protohominid. Its stature, shape and limb proportions are modelled on *A. afarensis* and the common chimpanzee. Its surface-area characteristics formed the basis for predictions about solar-heat gain and the respective contributions of evaporative and convective heat loss. This work suggested that bipedalism would have significantly reduced thermal stress on a 35-kg protohominid, and predicted that the drinking-water requirement for a bipedal hominid would have been 1.5 litres a day compared with 2.5 litres a day for a quadruped^{4,5}. Wheeler next tackled the physiological benefits and costs of hair loss, and showed that the advantages of a

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1. Berner, R. A. *Science* **249**, 1382–1386 (1992).
2. Clemens, S. C., Farrell, J. W. & Gromet, L. P. *Nature* **363**, 607–610 (1993).
3. Palmer, M. R. & Edmond, J. M. *Earth planet. Sci. Lett.* **92**, 11–26 (1989).
4. Raymo, M. E. & Ruddiman, W. F. *Nature* **359**, 117 (1992).
5. Sorkhabi, R. B. & Stump, E. *GSA Today* **3**, 85–92 (1993).
6. Edmond, J. M. *Science* **258**, 1594–1597 (1992).
7. Imbrie, J. et al. *Paleoceanography* **7**, 701–738 (1992).
8. Dia, A. N. et al. *Nature* **356**, 786–788 (1992).
9. Henderson, G. M., O'Nions, R. K. & Shackleton, N. J. *Eos* **74**, 176 (1993).
10. Hodell, D. A. & Woodruff, F. *Paleoceanography* (submitted).
11. Richter, F. M., Rowley, D. B. & DePaolo, D. J. *Earth planet. Sci. Lett.* **109**, 11–23 (1992).

naked skin for reducing dependence on evaporative cooling were obtainable only when radiant heat load was reduced, as is the case in a biped⁶. His model neatly accommodates the retention of head and shoulder hair, for they act to protect the skin area most exposed to direct solar radiation in a biped⁷.

The latest contributions move on from the more ape-like *A. afarensis* to a more human-like early African *Homo erectus* or *Homo ergaster*. Ruff⁸ had already pointed to the interaction between thermoregulation, climate and the relative linearity of body physique in these hominids. Wheeler construes the larger body size⁹ and taller physique¹ of early African *Homo erectus* as being an extension of the benefits of bipedalism. The larger and taller bodies reduce the relative demand for water and increase locomotor efficiency, thus, Wheeler claims, allowing the larger-bodied, taller hominids, to range more widely for food and other resources.

But is there not a flaw in this argument? If bipedalism was so beneficial for one savannah-dwelling primate, why is the other, the ubiquitous baboon, not bipedal? Wheeler argues that humans and baboons have different evolutionary histories, with that of humans providing an appropriate template for the anatomy needed by a biped. He has speculated that the large muzzles of baboons may be a mechanism to keep the brain's blood cool using a counter-current system and evaporation from the nasal mucous membranes, much as dogs do. Humans, and by inference early hominids, cannot selectively cool their brains and have to rely on whole-body cooling. Hence the premium on an efficient cooling system which uses the minimum of water. Wheeler's analysis is elegant. But does it account for the origins of hominid bipedalism, or merely explain why bipedalism was advantageous when hominids routinely foraged in more open savannah habitats?

Hunt's work^{2,10}, by contrast, is unashamedly analogical and comparative. He demonstrates that analogical reasoning as such suffers from no special flaws (what usually weakens such studies is their design), and did what we had all wrongly assumed had been done. He made a careful field study of the circumstances in which a close relative of modern humans, the common chimpanzee, assumes a bipedal posture and moves using a bipedal gait. More than 600 hours of observation of 26 adult chimpanzees in natural habitats produced unexpected results.

The vast majority, 80 per cent, of the bipedalism observed was postural and related to feeding. Bipodal postures were either strictly terrestrial, with the animals standing on the ground and reaching to feed, or occurred when animals stood on a slender branch and balanced themselves by raising an arm to clutch another

branch. Locomotion, by contrast, made up less than 4 per cent of bipodal activity, and even this consisted of rather ungainly shuffling between feeding sites. The larger the animal and the higher ranking the individual, the more likely it was to feed terrestrially using an upright posture. Thus common chimpanzees are refined postural bipeds, but not whole-hearted locomotor bipeds. They do not use their bipedalism in open habitats, but in the shade of the trees in which they feed.

Hunt not only provides invaluable field observations, but also exposes the fallacy of assuming that hominid bipedalism was an adaptation to a savannah environment; furthermore, his studies are a reminder of the usefulness of recognizing different types and levels of involvement in bipodal behaviours^{11,12}. Hunt defines two categories which he calls postural and locomotor bipedalism. For example, whereas he provides cogent functional anatomical arguments that *A. afarensis* was a postural biped, he suggests that an emphasis on locomotor bipedalism did not appear until the emergence of early African *Homo erectus*, or *Homo ergaster* around 1.9 million years ago¹³.

With the appearance of these new papers we have, as it were, taken a step forward. Hunt provides powerful evidence which integrates earlier ideas linking the emergence of bipedalism with either terrestrial^{12,14,15} or arboreal¹⁶ feeding postures. Wheeler shows that postural bipedalism could have been nudged towards locomotor bipedalism because of its advantages in maintaining heat and water balance in a savannah environment. This work goes well beyond the *Just-So* stage. Both hypotheses contain predictions, although we will need better information about early hominid habitat preferences and ranging behaviours in order to test them. The good news is that collaborations between palaeontologists and those involved in other methods of palaeoenvironmental reconstruction are beginning to make such data available¹⁷. □

Bernard Wood is in the Department of Human Anatomy and Cell Biology, The University of Liverpool, PO Box 147, Liverpool L69 3BX, UK.

1. Wheeler, P. E. *J. hum. Evol.* **24**, 13–28 (1993).
2. Hunt, K. D. *J. hum. Evol.* (in the press).
3. Lovejoy, C. et al. *Am. J. phys. Anthropol.* **38**, 757 (1973).
4. Wheeler, P. E. *J. hum. Evol.* **21**, 117–136 (1991).
5. Wheeler, P. E. *J. hum. Evol.* **23**, 91–98 (1992).
6. Wheeler, P. E. *J. hum. Evol.* **23**, 379–388 (1992).
7. Wheeler, P. E. *J. hum. Evol.* **24**, 23–28 (1985).
8. Ruff, C. B. *J. hum. Evol.* **21**, 81–105 (1991).
9. Wheeler, P. E. *J. hum. Evol.* **23**, 351–362 (1992).
10. Hunt, K. D. thesis, Univ. Michigan (1989).
11. Probst, J. J. *Am. J. phys. Anthropol.* **52**, 175–190 (1980).
12. Rose, M. D. in *Food Acquisition and Processing in Primates* (eds Chivers, D. J., Wood, B. A. & Bilsborough, A.) (Plenum, New York, 1984).
13. Wood, B. A. *Nature* **355**, 783–790 (1992).
14. Jolly, C. *J. Man* **5**, 1–26 (1970).
15. Wrangham, R. W. *J. hum. Evol.* **9**, 329–331 (1980).
16. Tuttle, R. H. *Phil. Trans. R. Soc.* **292**, 89–94 (1981).
17. Cerling, T. E. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **97**, 241–247 (1992).

High aspirations

As a helicopter takes off, the ground still feels its weight. Its undercarriage ceases to press on the ground, but the downwash of its rotor does so instead. The higher it rises, the more widely and shallowly is this excess air pressure distributed over the ground beneath; but it always adds up to the craft's full weight. The same is true of a fixed-wing aircraft.

So, says Daedalus, a high-flying plane could be detected, located and even weighed, by a big distributed web of sensitive barometers. Its height could be determined from the size of the high-pressure region it created, its speed and direction from the changing position of the centre of pressure, its weight and fuel consumption by the total force on the ground and its rate of decline. If it dropped a bomb or a parachutist, the integrated loss of ground pressure would give the weight of the released object. The implications for air-traffic control and military defence are obvious. The technique emits no radiation and cannot be detected, let alone jammed; a radar-defeating 'stealth' bomber would show up as clearly as any other plane. Only a stealth zeppelin, with military disadvantages of its own, could evade it.

Daedalus further argues that this total transfer of rigorously conserved momentum from aircraft to Earth, should be reversible. Imagine, he says, an array of upward-pointing blowers, spread over a huge area but with their axes all aimed towards a single point high in the sky. The air-momentum they project upwards will traverse an exactly converse path to that of an overflying plane. Rising upwards and inwards, it will ultimately converge on the target point. The resulting massive local upward momentum-flux could support a solid object suspended at that point.

And this is the basis of Daedalus's novel sky-platform. It is a flat 'flying saucer', levitated about 12 km up by an array of blowers distributed over the ground beneath it. At that height its horizon will be 400 km away. Far cheaper than any geosynchronous satellite, it will unify the telecommunications of an entire small country, or a whole region of a large one. It will act as the single central exchange for all the mobile phones of the region, and the single transmitter for all TV sets. They will need no elaborate aerials to receive its relatively local signal. It could also carry a surveillance platform, an air-traffic monitor and an astronomical and meteorological observatory. A simple control system could stabilize it in the sky by constantly adjusting the blowers so as to keep its radiated signal in the same apparent position.

David Jones

Legacy of mercury pollution

SIR — From about 1570, South and Central America established a hegemony on the silver market which lasted for more than 300 years^{1,2}. The primary impetus for the massive silver output was the introduction of a cheap and simple technology — the *patio* or mercury amalgamation — into silver production which was ideally suited for the low-grade ores (as low as 0.4 kg per tonne of ore) and some unique ore minerals (such as argentite and cerargyrite) common in the region. Although the *patio* process supplied the silver that sustained the European economy, it also left an unparalleled legacy of massive mercury pollution.

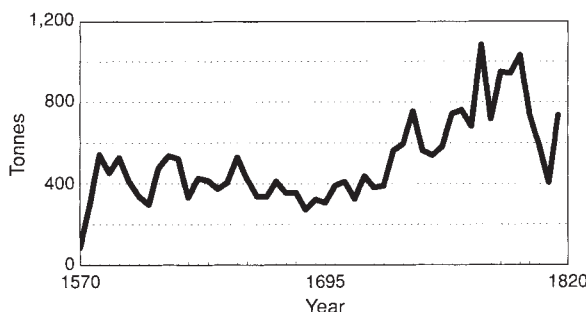
Although the principle of amalgamation had been known and used since ancient times³, its development into an industrial-scale operation was first made in New Spain (now Mexico) in 1554 by Bartolome de Medina^{1,2}. Even as late as 1870, about 70% of all the Mexican silver was still being produced by this process². Although it was supplanted by the 'barrel amalgamation' or Born process in the late nineteenth century, the technological nexus between silver and mercury was not severed until cyanide amalgamation was introduced around 1900 (see ref. 2).

Virtually all the mercury used in Spanish America came from three sources, in order of volume being Almaden in southern Spain, Huancavelica in central Peru and Idria in modern Slovenia³⁻⁵. Although some of the mercury used to extract the silver was recovered, a large fraction was generally wasted in the process because of the crude equipment and conditions. Until the middle of the eighteenth century, around 1.5 kg of mercury was lost for every kg of silver produced^{2,4}. The ratio (or *correspondencia*), however, could be as low as 0.85 kg Hg per kg Ag for impoverished ores and as high as 4.1 kg Hg per kg Ag for very rich ores³⁻⁵. Because of depressed mercury prices during 1760–1810, the loss of 2.4–2.9 kg Hg per kg Ag produced became common in many mining districts⁶. The *correspondencia* for the colonial silver mines were quite similar to the current loss of mercury associated with gold extraction in the Amazon of Brazil, typically 1.3–1.7 kg per kg of gold recovered^{6,7}.

Because nearly all the mercury produced in Almaden and Huancavelica went to the silver refineries in Spanish America, I have estimated the annual loss of mercury (see figure) using the outputs from these two sources and the

recorded imports from the Idria mines²⁻⁵ since the discovery of the Huancavelica mercury deposits in 1563. Between 1580 and 1820, the calculated losses varied from 292 to 1,085 tonnes per yr, with an average of 527. By comparison, the input of mercury into the Amazon associated with the current gold rush is 90–120 tonnes per yr (ref. 6). The cumulative loss of mercury in South America between 1570 and 1820 was about 126,000 tonnes (see figure).

About 99,400 tonnes of silver were produced in South and Central America



Mercury losses from the refining of silver in colonial South America. Virtually all the mercury produced from the Huancavelica and Almaden mines went to the silver mines of South America; the consumption and discharge of mercury each year is derived from the mercury output by the Huancavelica mines, 85% of the output by the Almaden, and any imports from the Idria mines. Based on various compilations, especially those in refs 2–7, 9–12.

between 1820 and 1900 (refs 8, 9). Assuming the ratio of mercury lost to silver produced to be 1:1 (less than the ratios in colonial times) and that 70% of the silver was recovered by the *patio* process and its modifications, the cumulative discharge of mercury during the 80 years is about 70,000 tonnes. From the total figure, the average discharge rate in post-independence times is estimated to be 875 tonnes per yr. Thus, for 1570–1900, when the *patio* process was in common use, the total discharge of mercury from silver mining in South and Central America was around 196,000 tonnes.

Although mercury was used in numerous silver mines, the most sustained losses occurred in only a few major silver-mining regions⁵. What happened to the unprecedented quantities of mercury discharged? The old Spanish literature is virtually silent on the ecological and human health effects of what would have been severe mercury pollution. Around 10% of the mercury supply would have been lost during transport and storage^{1,2} and about 25–30% of the silver (and implicitly the mercury as well) would have been left behind in the residue or removed in waste streams². The balance of the mercury used (60–

65%) would have been released to the atmosphere during the burning of the mercury amalgam, the amalgamation process on the open *patio* floor or in heated cauldrons, and the squeezing of the *pella* (amalgam) to remove the excess mercury. The fraction estimated to be emitted to the atmosphere in colonial times is comparable to the 65–83% for current recovery of gold in the Amazon^{6,7}.

Using these data, the atmospheric fluxes of mercury from the silver mining in colonial South America during 1587–1820 would have been 180–705 tonnes per yr. Because the anthropogenic sources of the period released much less than the total 910–6,200 tonnes Hg emitted annually by modern industries¹⁰, it is clear that the silver mines were the dominant source of atmospheric mercury pollution. The importance of this source of mercury pollution has not been considered in previous discussions of the global and regional cycling of mercury¹¹. Under the hot tropical conditions especially of Mexico, any mercury in the abandoned mine wastes or deposited in the aquatic sediments remains liable to be methylated and released to the atmosphere¹². And any deposited mercury can subsequently become mobilized and could cycle in the atmosphere for a long time. It is therefore possible that the Spanish American silver mines were partly responsible for the high background concentrations of mercury in the global environment now being reported.

Jerome O. Nriagu
Environment Canada,
National Water Research Institute,
Box 5050, Burlington,
Ontario L7R 4A6,
Canada

- Prieto, C. *Mining in the New World* (McGraw-Hill, New York, 1973).
- Brading, D. A. & Cross, H. E. *Hispanic Am. Hist. Rev.* **52**, 547–579 (1972).
- Blanchard, I. *Russia's Age of Silver* (Routledge, London, 1989).
- Fisher, J. R. *Silver Mines and Silver Miners in Colonial Peru, 1776–1824* (Centre for Latin American Studies, Univ. Liverpool, 1977).
- Bethell, L. (ed.) in *The Cambridge History of Latin America* 105–151 (Cambridge Univ. Press, 1984).
- Pfeiffer, W. C. et al. *Sci. tot. Envir.* **87**, 233–240 (1989).
- Lacerda, L. D. & Salomons, W. *Mercury in the Amazon* (Dutch Ministry of Housing, Physical Planning & Environment Report, Inst. of Soil Fertility, Haren, 1991).
- Mohide, T. P. *Silver* (Ontario Mineral Policy Background Paper No. 20, Ministry of Natural Resources, Toronto, 1985).
- Cronshaw, H. B. *Silver Ores* (Murray, London, 1921).
- Nriagu, J. O. & Pacyna, J. M. *Nature* **333**, 134–139 (1988).
- Andren, A. W. & Nriagu, J. O. in *Biogeochemistry of Mercury in the Environment* (ed. Nriagu, J. O.) 1–21 (Elsevier, Amsterdam, 1979).
- Lindberg, S. E. & Turner, R. R. *Nature* **268**, 133–136 (1977).

Loss of kinase activity

SIR — Vetrie *et al.*¹ showed that a novel intracellular protein-tyrosine kinase is involved in X-linked agammaglobulinaemia (see also ref. 2). They related two point mutations which cause Lys 430→Glu and Arg 525→Gln exchanges to loss of kinase function and in turn to the expression of the immune deficiency disease.

Our own data provide a basis specifically to assess the function of the residues Lys 430 and Arg 525 in the newly discovered *atk*-tyrosine kinase. Recently, we solved the 2.0-Å crystal structure of the protein kinase A catalytic subunit from porcine heart in a complex with a nucleotide triphosphate and a pseudo-substrate³, and suggested a mechanism for phosphotransferase and substrate binding of protein kinases. Residue Lys 72 of protein kinase A corresponds to Lys 430 in *atk* and is invariant in all protein kinases⁴. This residue is involved in MgATP binding⁵, and a direct role in catalysis has often been proposed. We found, however, that Lys 72 is too far away from the catalytic site to participate in the immediate catalytic act, but the residue does bind MgATP, as it coordinates oxygens of the α - and β -phosphoryl groups. A glutamate substitution is likely seriously to interfere with ATP coordination.

Carrera *et al.*⁶ proposed a seemingly contradictory role of the conserved lysine in catalysis and not in ATP binding, mainly because the affinity of 8-azido- $[\gamma\text{-}^{32}\text{P}]$ ATP for the tyrosine kinase pp56^{lck} was not significantly changed after mutating the lysine to arginine, whereas kinase activity was lost. However, the arginine must not necessarily affect the affinity for the triphosphate, but could critically disturb its stereochemistry, leading to dislocation of the γ -phosphate.

Residue Lys 168 of protein kinase A, which has not yet been considered to have a general role in catalysis as it is conserved only in Ser/Thr-protein kinases, appears to have an important function in the immediate process of phosphotransfer through stabilizing the transition state. Its positive charge is located within hydrogen bonding distance of the γ -phosphate and in the vicinity of the substrate hydroxyl group. In tyrosine kinases two sequences are found at this relative position, either Arg(168)-Ala-Ala, or Ala(168)-Ala-Arg (ref. 4). Because an extended residue at position 170 (corresponding to Arg 525 in *atk*) would place its charge in a position occupied by the Lys 168 charge in protein kinase A if the position corresponding to 168 is small and hydrophobic, it is likely that this spatial position of a positive charge is conserved among all protein

kinases. The correct alignment as given in ref. 4 should therefore not be adjusted to align the alanine pair into homologous positions.

We suggested that in tyrosine kinases the Lys 168-homologue is functionally replaced by an arginine. The important functions of these residues in ATP binding and in the catalytic process, respectively, explain the loss of protein-kinase activity in Lys 430 and Arg 525 mutants of the *atk* kinase.

Dirk Bossemeyer

Department of Pathochemistry,
German Cancer Research Centre,
D-6900 Heidelberg, Germany

1. Vetrie, D. *et al.* *Nature* **361**, 226–233 (1993).
2. Tsukada, S. *et al.* *Cell* **72**, 279–290 (1993).
3. Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H. & Huber, R. *EMBO J.* **12**, 849–859 (1993).
4. Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42–52 (1988).
5. Zoller, M. J. & Taylor, S. S. *J. biol. Chem.* **254**, 8363–8368 (1979).
6. Carrera, A. C., Alexandrow, K. & Roberts, T. M. *Proc. natn. Acad. Sci. U.S.A.* **90**, 442–446 (1993).

No HMG-1 box signature

SIR — There have been several recent reports of the cloning and sequencing of a complementary DNA encoding a protein, CCG1, which is thought to be involved in the progression of cells through G1 phase of the cell cycle¹ and which has been shown to be one of the TATA-binding protein (TBP)-associated factors (TAFs) in transcription initiation complexes in humans^{2,3}. Indeed, a functional homol-

ogous list obtained using software such as the Blast network service at the NCBI (ref. 8).

I have examined the human CCG1 gene product sequence by several methods. An initial screening identifies several regions of low compositional complexity (regions of locally 'biased' amino-acid composition such as polyglutamine and basic regions), including the central region of the proposed HMG-1 box in CCG1 (residues 1,165–1,205)⁹. Furthermore, this same region is a statistically significant mixed-charge segment¹⁰. This would explain the initial erroneous statement that this sequence is similar to the charged HMG-1 box domain. A search of the NCBI non-redundant protein sequence database using Blast software⁸ reveals that the only domain with any similarity to other proteins in the database is the region between residues 1,336 and 1,529, which contains two repeats of a previously identified bromodomain^{3,11}. Regions in this domain show similarity to several proteins including GCN5, SPT7, STH1, NPS1, SNF2/GAM1 and YK107 from *Saccharomyces cerevisiae*; human peregrin and RING3; and *fsh* from *Drosophila*. No statistically significant similarities with any of the numerous (more than 130) HMG-1-box-containing proteins in the database are detected. There are also two repeats of the bromodomain in the *Drosophila* TAF_{II}250 protein⁴.

These findings do not support the hypothesis that there is an HMG-1 box domain in these proteins or that this is the domain that has DNA-binding capabilities. In addition, CCG1 protein has also

[GSA][YF]..[YFW].*[GSA]..[WYF].....[KRQ]..[YFW].....[KRQ]..[YFH].....[YFW]

ogue of this protein, TAF_{II}250, has been isolated from the *Drosophila* TFIID complex⁴. The current model for the function of this *M_r* 250,000 protein is that it is a central core for several protein-protein interactions with other TAF proteins⁴. I was surprised to find that the CCG1 protein does not contain an HMG-1 box domain, as previously noted¹.

HMG-1 box domains have been found in many proteins involved in transcription regulation as well as in other cellular functions^{5–7}. HMG-1 box domains usually contain a regularly spaced pattern of aromatic residues with no gaps needed for alignment. Furthermore, at a variable distance towards the amino-terminal end of the domain, there is a hydrophobic region in which at least two out of five amino acids are aromatic. This is best represented by the regular expression or signature shown in the box above⁷.

A search of the protein sequence databases using this sequence as the query gives a list of proteins containing an HMG-1 box domain, which agrees with a

been reported to share sequence similarity with NF- κ B, SWI4 and bacterial σ -factors³. None of these similarities is significant when analysed by quantitative methods.

David Landsman

National Center for Biotechnology
Information,
National Library of Medicine,
National Institutes of Health,
Building 38A, Room 8N-807,
Bethesda, Maryland 20894, USA

1. Sekiguchi, T., Nohiro, Y., Nakamura, Y., Hisamoto, N. & Nishimoto, T. *Molec. cell. Biol.* **11**, 3317–3325 (1991).
2. Ruppert, S., Wang, E. H. & Tjian, R. *Nature* **362**, 175–179 (1993).
3. Hisatake, K. *et al.* *Nature* **362**, 179–181 (1993).
4. Weinzierl, R. O. J., Dynlacht, B. D. & Tjian, R. *Nature* **362**, 511–517 (1993).
5. Ner, S. *S. Curr. Biol.* **2**, 208–210 (1992).
6. Lilley, D. M. *Nature* **357**, 282–283 (1992).
7. Landsman, D. & Bustin, M. *BioEssays* (in the press).
8. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. *J. molec. Biol.* **215**, 403–410 (1990).
9. Wootton, J. C. & Federhen, S. *Computers Chem.* (in the press).
10. Brendel, V., Bucher, P., Nourbakhsh, I. R., Blaisdell, B. E. & Karlin, S. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2002–2006 (1992).
11. Haynes, S. R. *et al.* *Nucleic Acids Res.* **20**, 2603 (1992).

Neanderthals rehabilitated

Ian Tattersall

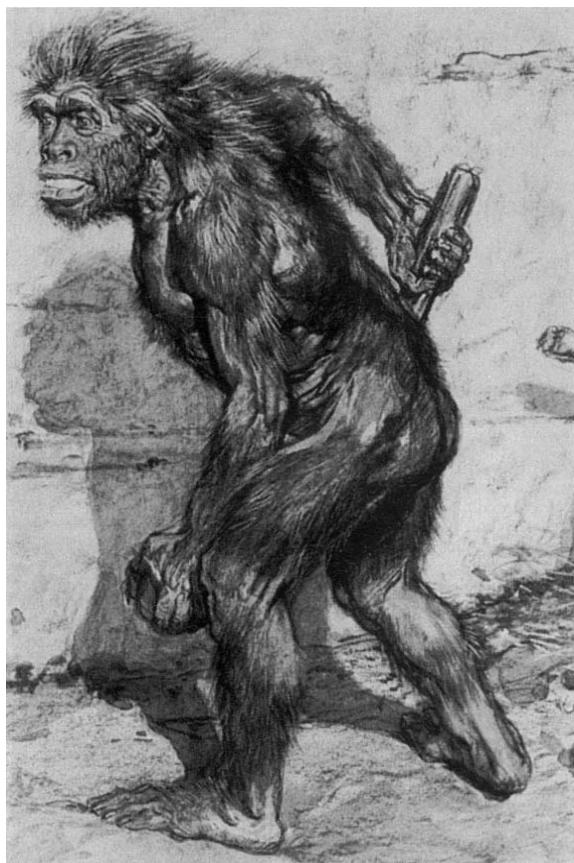
In Search of the Neanderthals: Solving the Puzzle of Human Origins. By Christopher Stringer and Clive Gamble. *Thames and Hudson*: 1993. Pp. 247. £18.95, \$29.95.
The Neanderthals: Changing the Image of Mankind. By Erik Trinkaus and Pat Shipman. *Knopf/Jonathan Cape*: 1993. Pp. 452. \$30, £20.

NEANDERTHAL. There's probably no more evocative word in the lexicon of science, and certainly none that's more redolent in the public mind of brutish benightedness. This undeserved reputation is the price that the Neanderthals have paid for being the first extinct human relatives to be discovered, and it's particularly unfair because these now-vanished people clearly resembled their relative *Homo sapiens* more closely than any other extinct human species we know of. But perhaps there's hope, for two leading specialists have now taken it upon themselves to rehabilitate the public image of the Neanderthals in books that are aimed at a general readership but which will also, in very different ways, be of value to professional palaeo-anthropologists.

In this laudable effort, each has conscripted a coauthor. Chris Stringer, the best-known exponent of the 'replacement hypothesis' of modern human origins, has joined forces with the Palaeolithic archaeologist Clive Gamble. Erik Trinkaus, whose interests lie principally in Neanderthal functional anatomy, has teamed up with the taphonomist and science writer Pat Shipman, whose journalistic skills are evident in a highly readable end result. The books are largely complementary, and could hardly be more different (the two teams even disagree on the spelling of 'Neanderthal', Stringer and Gamble evidently stressing, rightly, that the term had become part of the English language long before the Germans dropped the 'h').

Trinkaus and Shipman take a strictly historical approach, proceeding chronologically from the discovery of the original Neanderthaler in 1856 up to the present day, with digressions back to the earliest intimations of human antiquity and the roots of geological and evolutionary thought in the late eighteenth century. Their account not only provides a more or less complete history of the interpretation(s) of the accumulating human fossil record, but it effectively places changing views in the context of evolving political and social circumstances. Although it contains scat-

tered minor inaccuracies, this is an authoritative record, and particularly useful in bringing the story more up to date than any previous effort. It's also diplomatic. The potted biographies of important figures with which the narrative is peppered become more hagiographical as we



Brutish benightedness — Kupka's 1909 reconstruction of a Neanderthal, based on Marcellin Boule's scientific study of the Chapelle-aux-Saints skeleton and published in *L'Illustration*.

approach the present day: there will be few ruffled feathers among the featured members of the "gifted generation" of Neanderthal experts that is currently in full squabble, although certain absent noses may be put a little out of joint.

Curiously enough, even though Trinkaus's own mini-biography describes him as distinguished from the crowd by his concentration on the lives rather than the fate of the Neanderthals, the matter of Neanderthal behaviour and lifestyle is broached systematically only in the last

half-dozen pages of his book. If you want to know in detail what it was like to be a Neanderthal, you have to turn to Stringer and Gamble. Their volume is altogether tougher sledding, but it will richly reward your concentration. History is skipped over lightly (although not so lightly that the sources of the historical burden borne by the Neanderthals don't become apparent); instead, the book targets a set of particular issues: models of human evolution, late Pleistocene chronologies and environments, Neanderthal origins, biology, technology and behaviour, the origin and spread of *Homo sapiens*, and so forth.

The breadth of the resulting picture of the Neanderthals is a dramatic testimony to how valuable — indeed, essential — is close collaboration between palaeoanthropology and archaeology in reconstructing the lives and histories of our toolmaking precursors. General readers may find the discussion rather hard going, while specialists may wish for more detail in some areas (despite a spectacularly useful if eye-straining chronological appendix of sites); but this is an unprecedentedly well-rounded portrait of an extinct human species, and one that gains enormously from a detailed comparison of inferred Neanderthal behaviours and capacities with those of the early moderns who replaced them. Not everyone will agree with all of the inferences made here, but at the very least the appropriate questions get a much-needed airing.

So — to get to the inevitable question — what did happen to the Neanderthals? Stringer and Gamble are pretty categorical that "Neanderthals did not evolve into modern Europeans", while Trinkaus and Shipman prefer to talk more vaguely about a "mosaic of local evolution, migration, admixture, absorption or local extinction . . . over at least 10,000 years". No agreement here (and none likely soon).

Yet this is the least of the differences between these two books, which are essentially not in competition with each other. If you want to learn a great deal about how palaeoanthropology got to where it is today, read Trinkaus and Shipman. If your interest is in knowing what can reasonably be said about Neanderthal origins, biology and behaviour, consult Stringer and Gamble. Better still, read both. □

Ian Tattersall is in the Department of Anthropology, American Museum of Natural History, New York, New York 10024-5192, USA.

Tesla's sparks of imagination

THE fledgling broadcasting industry of the early 1900s was blighted by fierce patent litigations. Patents in this highly innovative field were often loosely formulated and most wireless systems contained devices whose patents were held by several inventors or entrepreneurs. One of the most charismatic of these people was Nikola Tesla (1856–1943), an American born in Croatia of Serbian parents, who briefly worked for Edison and Westinghouse before carving out a career as a freelance inventor. It is generally known that he invented the induction motor based on the rotating magnetic field and the alternating-current polyphase power distribution system. He was assisted in this task by Westinghouse but hindered by Edison who had committed himself to direct current. During the "battle of the systems", Edison successfully lobbied New York State to adopt alternating current for the electric chair, and then pointed out in mock horror the deadly nature of these currents. Tesla fought back just as ferociously, won the battle for alternating current supply, and became the darling of New York society for his 'magical' experiments with powerful high-frequency currents.

It is far less well known that until 1943 Tesla, and not Marconi, was recognized by the US Supreme Court as having priority in the invention of radio. Recently, a remarkable legal document has surfaced that places this research in the context of Tesla's work on radio technology and electromagnetic radiation and propagation. This document, never intended for publication, is the transcript of a pre-hearing interview with Tesla by his legal counsel in 1916 to prepare themselves before he pressed his claims against the Marconi Company, and also to protect his own patent interest when called as an expert witness. The transcript has been edited by Leland I. Anderson and now appears in *Nikola Tesla on his Work with Alternating Currents and their Application to Wireless Telegraphy, Telephony, and Transmission of Power* (Sun Publishing, Denver, Colorado 80219, USA, \$40, pp. 237 (pbk)).

Tesla gives a fascinating account of the stages that led him in the 1890s to attempt to transmit electrical power through the earth without the use of wires. He developed spark and continuous-wave transmitters (installed on US Navy ships before the First World War), but in the end he almost perversely rejected wireless transmission by hertzian waves as too wasteful of energy, and opted instead for a system of worldwide communication by conduction through the earth. After initial experiments at Colorado Springs, Colorado, he unsuccessfully developed a commercial station in 1912 at Wardencliff, Long Island. He constructed a 167-foot tower transmitter (shown here) that end-

ed in a large metal-clad wooden sphere and which was connected to the earth by a 60-foot shaft through which the current passed. The electrical potential achieved was so great that the tower antenna had to be separated from its power plant by 350 feet, lest sparks should jump from the tower to the chimney of the power plant. This structure is described in detail in the second document reproduced in this volume, a legal document in which Tesla appealed in 1916 against the Wardencliff foreclosure judgement instigated by his debtor, George C. Boldt of the Waldorf-Astoria. This extraordinary structure was destroyed the previous year so that the property could be developed commercially. With it went Tesla's dream of worldwide wireless telephony.



Tesla was one of the most talented designers of electrical apparatus of his generation — some (including Tesla) would argue that he was the most prophetic and innovative — yet he never fulfilled his true potential. He received many honours and was twice nominated for the Nobel prize: in 1912 with Edison (Tesla refused to be associated with his old adversary) and in 1937 in his own right. In his final years, Tesla was a recluse, living in a succession of New York hotel rooms with only pigeons for company. He died an eccentric, embittered man. His papers were seized by the Alien Property Office and are now in the Nikola Tesla Museum in Belgrade. The unit of magnetic induction, the tesla, was named in his honour, and reminds us of one of the masters of an heroic epoch of electrical technology.

Willem Hackmann

Willem Hackmann is at the Museum of the History of Science, Old Ashmolean Building, Broad Street, Oxford OX1 3AZ, UK.

Techno-bonds

Jack Ruina

The Cold War and American Science: The Military-Industrial-Academic Complex at MIT and Stanford. By Stuart W. Leslie. Columbia University Press: 1993. Pp. 332. \$48.50, £28.

THE Second World War tremendously increased the importance of science and technology for the US military. After all, it was physical scientists at the forefront of their fields who successfully developed the atomic bomb and radar, achievements not forgotten by military establishments when the war ended. Afterwards, the US military and leading research universities developed a symbiotic relationship, funds flowing one way, relevant research results and scientific expertise the other. The Cold War, with its emphasis on technological superiority, intensified and bonded this relationship. The military became the primary sponsor of many fields of applied science, particularly guidance and control, electronics and materials.

Stuart Leslie, a young historian of science and technology, tells one part of this interesting and important story. He concentrates on the Massachusetts Institute of Technology (MIT) and Stanford University, which early on had achieved prominence in fields of interest to the military and then capitalized on the availability of military funding to become outstanding, world-class universities in applied science. He shows how key people with the technical competence, experience, vision and entrepreneurship developed projects and attracted funds.

Before the war, foundations and private companies were the principal sponsors of university research. After the war, military funds stimulated a huge increase in the amount of research done on campuses. The temptations for growth, for acquiring costly equipment, for graduate student support and for the opportunity to work on technically challenging problems were impossible to resist. If university scientists were to continue at anything like the level of their wartime work, there was no alternative but to depend on military funds.

It was the US Department of Defense that got the lion's share of government funds for research and development and could keep political interference in the disbursement of these funds at a minimum by invoking 'national security'. Most important, the military had the keenest interest in advanced technology; other possible government sponsors had neither the funds nor an interest in high technology. And soon after the war, the Office of Naval Research established the pattern for a fruitful government relationship with

universities. To this day, military sponsors offer universities greater freedom and fewer bureaucratic hurdles than most other government sponsors.

Leslie suggests that university research activities were unduly influenced by military priorities, and that this led not only to the neglect of other more worthy societal needs but also to the surrender of much of the freedom that constitutes a university's essence — a faustian bargain had been struck. But Leslie fails to consider the real dilemma faced by universities after the war. The military was the only provider of substantial funds for research and graduate education at the cutting edge of applied science. Had universities forgone this support, they would have remained in the backwaters of technology, leaving all the intellectual excitement of post-war electronics and aerospace technology to industry alone. In fact, MIT made valiant efforts to start large research programmes on energy and the environment. Although the institute was willing, government funding proved to be weak.

Leslie also neglects to describe how military-sponsored research since the war, particularly at MIT and Stanford, has been the source of some of the most important nonmilitary technological advances in computer science, solid-state electronics, microwave techniques, guidance and navigation systems and more. The students and staff of these institutions have become leaders of much of US civilian high-tech industry.

Leslie focuses on the 'cooption' aspects of accepting military support. The military certainly affected university research, but university researchers also affected military policy and not always to the liking of the military. The universities did not quite become handmaidens of the military. For instance, MIT — referred to in some Washington circles as the "left bank of the Charles River" — received special prominence on President Richard Nixon's "enemies list" merely because of the active dissent that arose at the institute over his military policies. Many faculty members, particularly from MIT and Stanford, were key figures over many years in promoting nuclear arms control and, because of their expertise in military technology, were among the most credible and convincing public witnesses in testifying about US and Soviet nuclear weapons.

All in all, the book is admirably researched, well documented and well written. Leslie's message has substance and is not shrill. But the definitive story of military and university research in the post-war years must take account of a fuller and more complex set of issues. □

Jack Ruina is in the Department of Electrical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.



MOUNTAINS of 'yellow gold' — every day, about 100 men brave gases to dislodge sulphur from the Kawah Ijen volcano in Java, Indonesia. Most of the sulphur is used for the bleaching of cane sugar. This picture is taken from *World Press Photo 1993*, a collection of the 180 or so prizewinning submissions to the 1992 competition for international photographic reporting. Thames and Hudson, £8.95 (pbk).

Dreaming on

Graham Ross

The End of Physics: The Myth of a Unified Theory. By David Lindley. *Basic Books*: 1993. Pp. 275. \$25.

THE End of Physics is concerned with the achievements and aspirations of physicists. It starts with a lightning review of the successes of mathematical science in explaining the physical world around us, from newtonian gravity and Maxwell's theory of electromagnetism to relativity, quantum mechanics and their merger in quantum field theory, the cornerstone of modern physics at the forefront of particle physics. All this leads the author to echo Einstein and Wigner's question: How can it be that maths is the correct description for objects of reality?

This is the prevailing theme of the book, and the basic question posed is whether mathematics has so dazzled physicists that they are now hell-bent down a blind alley. Lindley muses that theoretical physicists now indulge in recreational mathematics, which is aesthetically attractive but without predictive power. This is his "end of physics": the search for the mythical Theory of Everything, which offers explanations for everything we see around us but can be neither tested nor disproved.

I find Lindley's case unconvincing. He misses much of the thrust of modern research, which continually seeks to confront theory with observation. Far from this process running out of steam, we may be entering an exciting phase in the development of fundamental theory, in which the symbiosis of theory and experiment is likely to see further substantial progress in our understanding of the physical world.

Where then does the book go astray? Lindley starts nicely enough by illustrating the successes of mathematical science in classical physics. Maxwell's theory of electromagnetism is cited as an example of what is, today, seen as an elegant theoretical structure unifying the forces of electricity and magnetism. But to many it initially seemed to be an inexplicable mathematical abstraction. It was the fact that Maxwell's theory made testable predictions that proved to be true that led to the acceptance of the theory, not its underlying elegance *per se*. Lindley's description of other major developments in physics, including quantum mechanics, atomic physics and general relativity, provides a readable, if fairly dense, survey of some of the triumphs of mathematical physics.

The thread of Lindley's argument is lost when he tries to describe modern developments in particle physics and the field-theoretic descriptions of the fundamental

interactions. He does not seem fully to appreciate the enormous achievement of the past 40 years, which has culminated in the Standard Model. This model provides a fully relativistic quantum theory of the electromagnetic, weak and strong interactions that predicts physical phenomena with incredible accuracy. But Lindley gives the impression that this theory of particle physics is a mess, and that it is necessarily so because that is the way the world appears to work. Thus, we are told: "The quark model and the theory of electroweak unification may be neither the most obvious nor the most beautiful of intellectual constructions", despite the fact that they have elegantly described a plethora of states and their interactions in terms of a simple set of fundamental states, with interactions dictated by a generalization of the universal 'gauge' principle implicit in Maxwell's theory. The beauty and simplicity of the theory lies in an appreciation of the symmetries of the system, the patterns that relate apparently dissimilar particles and forces. Perhaps more than anything else this appreciation has shaped the way we think of the modern world of subatomic physics. It is not, as Lindley would have us believe, that simplicity has become the religion of the modern physicist, but rather that the experimental success of theories based on recognizing this simplicity has led us to an understanding of the importance of symmetry.

Unfortunately, this lack of understanding of the status of modern physics pervades the rest of the book. We are given no sense of the motivation behind the search for Grand Unification, which seeks to explain some of the questions left unanswered by the Standard Model by looking for larger symmetries. The author argues that such theories are in limbo with "no prospect of doing experiments to check it in even the barest detail". This blithely ignores one of the most important predictions of the theory of Grand Unification, which relates the strengths of the fundamental interactions and has been experimentally tested in detail in recent years following the precision measurement of these couplings at the accelerators at CERN (European Laboratory for Particle Physics) and elsewhere. The fact that one of the simplest Grand Unified schemes gives a prediction that is in excellent agreement with experiment is one reason why such theories are taken seriously.

The confusion remains in other parts of the book. In dealing with cosmology, Lindley introduces us to the idea of inflation, a period of rapid growth in the early Universe caused by a phase transition thought to have occurred shortly after the Big Bang. He ascribes this hypothesis to an "even more extravagant use of Grand Unified theories". But phase transitions

abound in physical systems and one does not need Grand Unification to motivate one to consider this possibility in the early Universe. It is true that the field-theoretic use of a phase transition to give mass to the W and Z bosons is an untested aspect of the Standard Model, but that is one of the central reasons for building the new accelerators at CERN and in Texas: namely, to test the mechanism responsible for the origin of mass. Further, Lindley does not even discuss one of the most important implications of inflation. In addition to explaining the large-scale smoothness of the Universe, it also predicts that inevitable quantum fluctuations will generate inhomogeneities at a low level. The recent measurements of inhomogeneities by the Cosmic Background Explorer (COBE) allow us to use observation to probe the structure of the Universe during the inflationary era.

Of course, as Lindley notes, cosmological models are so far only hypotheses. The subject is young and physicists are still trying to build a viable theory. Far from having reached the end of physics by building the myth of an untestable unified theory, we are at a profoundly exciting stage in which observation, both astronomical and in the laboratory, offers the prospect of new insights into the nature of physical law. □

Graham Ross is in the Department of Theoretical Physics, University of Oxford, 1 Keble Road, Oxford OX1 3NP, UK.

Psycho-analysis

Solomon H. Snyder

Molecules and Mental Illness. By Samuel H. Barondes. W. H. Freeman/Scientific American Books: 1993. Pp. 216. \$32.95, £18.95.

No field of medicine has been so radically transformed in the modern era as psychiatry. In the late nineteenth century, giants such as Emil Kraepelin made astute clinical observations to delineate what we now call schizophrenia and manic-depressive illness. Similar descriptive medical approaches led to such breakthroughs as the discovery of bacteria as the cause of certain diseases. Throughout the twentieth century, psychiatry lacked comparable fundamental insights. As a result of its domination by psychoanalysts in the United States, the field lost much of its link with biology and the disease concept of mental illness. But with the advent of psychotherapeutic drugs in the past 30 years, biological psychiatry has re-emerged as a dominant force; we now have a much better understanding of the molecular basis of synaptic transmission

and drug action and of the genetic mechanisms that may underlie the main mental illnesses.

In an exquisitely elegant volume, Samuel Barondes chronicles these changes in psychiatry and provides rigorous yet highly readable explanations of the biological underpinnings of mental illness. Barondes is uniquely qualified for the task. Fully trained in psychiatry, he has a long record of important advances in molecular neuroscience and is currently the director of the department of psychiatry at the University of California, San Francisco, while still maintaining a research laboratory.

Barondes looks at the beginning of biological psychiatry, describing how Sigmund Freud was himself trained as a neurobiologist. Years after elaborating the theory of psychoanalysis, Freud still regarded the underlying causes of the main emotional disorders as biological. Two chapters are devoted to genetics, including the genetic basis of illnesses such as Huntington's disease as well as a reasonably thorough description of the molecular biology of genes. There follow chapters on neurotransmitters, receptors and actions of the principal psychiatric drugs. In three separate chapters, the author deals with the main psychiatric illnesses: schizophrenia, manic-depressive illness and obsessive-compulsive disorder. The final chapter is a 'recapitulation' of the entire book in verse.

Throughout, Barondes carefully balances clinical descriptions with the biological features. He creates a better feel for emotional distress than most psychiatry texts by providing extensive accounts of different cases, including recollections by patients of their own disturbances. This mixture of vivid clinical portrayals with molecular mechanisms goes a long way to dissipate the conceptual tension between organic and psychological approaches to psychiatry. Although the author is committed to an organic aetiology for the principal psychiatric conditions, he appreciates their psychodynamic aspects and the role of psychotherapy.

The volume is written in a lively, lucid style that makes complex ideas eminently accessible. Like others in the Scientific American Library series, the book is superbly illustrated with diagrams clarifying scientific concepts as well as photographs and portraits ranging from Howard Hughes at the controls of his aeroplane to a mildly surrealistic portrait of Sigmund Freud by Ben Shahn. It is an important contribution that will interest all thoughtful readers, whether scientists or educated lay people. □

Solomon H. Snyder is in the Department of Neuroscience, School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore, Maryland 21205-2185, USA.

Crystal structure of cyclin-dependent kinase 2

Hendrik L. De Bondt*, Jody Rosenblatt†, Jarmila Jancarik*, Heather D. Jones†, David O. Morgan† & Sung-Hou Kim*‡

* Department of Chemistry and Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, USA

† Department of Physiology, University of California, San Francisco, California 94143-0444, USA

Cyclin-dependent kinase 2 (CDK2) is a member of a highly conserved family of protein kinases that regulate the eukaryotic cell cycle. The crystal structures of the human CDK2 apoenzyme and its Mg^{2+} ATP complex have been determined to 2.4 Å resolution. The structure is bi-lobate, like that of the cyclic AMP-dependent protein kinase, but contains a unique helix-loop segment that interferes with ATP and protein substrate binding and probably plays a key part in the regulation of all cyclin-dependent kinases.

THE cyclin-dependent protein kinases (CDKs) are crucial regulators of the timing and co-ordination of eukaryotic cell cycle events¹. Transient activation of these kinases at specific cell cycle stages is thought to trigger the principal transitions of the cell cycle, including 'Start' (when the cell becomes committed to a new division cycle) and entry into mitosis. In yeast, genetic evidence suggests that both of these transitions are controlled by a single CDK (CDC2 in *Schizosaccharomyces pombe* and CDC28 in *Saccharomyces cerevisiae*)². In human cells, cell cycle events are governed by several CDKs. Two of these proteins (known as CDC2 and CDK2) have been studied extensively. Both are closely related to yeast CDC2/CDC28 (60–65% identity in amino-acid sequence) but appear to be functionally distinct. Human CDC2 has been implicated mainly in the control of mitosis¹, whereas CDK2 may be involved in the control of G1 and S phase events^{3,4}.

Cell-cycle dependent oscillations in CDK activity are induced by complex mechanisms that include binding to positive regulatory subunits and phosphorylation at positive and negative regulatory sites. CDK2 and CDC2 are small proteins ($M_r \sim 34$ K) that contain little more than the conserved catalytic core found in all eukaryotic protein kinases⁵. They are inactive as monomers, and activation requires binding to cyclins, a diverse family of proteins whose levels oscillate during the cell cycle⁶.

Kinase activity of the CDK–cyclin complex is extensively regulated by phosphorylation. Cyclin binding alone does not fully activate the CDK catalytic subunit, although in some cases partial activation can occur^{7,8}. After cyclin binding occurs, a separate protein kinase, known as the CDK-activating kinase, phosphorylates the CDK subunit on a threonine residue (Thr160 in human CDK2 and Thr161 in human CDC2)^{7,9,10} (D. Desai and D.O.M., unpublished results). Phosphorylation at this site leads to maximal activation of the CDK–cyclin complex^{7–11}. Under some circumstances, CDK2 and CDC2 can also be negatively regulated by phosphorylation on Tyr15 and to a lesser extent on the adjacent threonine residue (Thr14). Phosphorylation at one or both of these sites inhibits the kinase activity of the Thr160/161-phosphorylated CDK–cyclin complex^{9,10,12–15}.

The control of the cell cycle by cyclin-dependent kinases thus involves a complex array of regulatory mechanisms. To begin to understand these mechanisms, we have carried out a crystallographic analysis of the structure of the human CDK2 catalytic subunit. This is the first member of the CDK protein kinase subfamily for which detailed structural information is available.

So far the only other protein kinase structure known is that of the catalytic subunit of mouse cyclic AMP-dependent protein kinase (cAPK), which was crystallized in a complex with a pseudo-substrate inhibitor peptide^{16–19}. CDK2 and cAPK are members of distantly related protein kinase subfamilies that employ distinct regulatory mechanisms: whereas the CDK2 apoenzyme is inactive and requires positive regulatory subunits and phosphorylation for activity, the cAPK catalytic subunit is fully active by itself, but inhibited by association with a negative regulatory subunit. Thus, our CDK2 structure provides new insight into the molecular mechanisms underlying protein kinase regulation.

Structure determination

We determined the 2.4 Å resolution structure of the CDK2 apoenzyme by the multiple isomorphous replacement (MIR) method, as well as that of the CDK2– Mg^{2+} ATP complex by difference Fourier analysis. The CDK2 protein was purified by mild chromatographic procedures from lysates of insect cells infected with a recombinant baculovirus^{7,20}. This protein is not associated with cyclin and is not phosphorylated, and as a result its activity is undetectable in kinase assays with standard substrates such as histone H1. The purified protein can be fully activated when complexed with purified cyclin A and phosphorylated on Thr160 by the CDK-activating kinase (D. Desai, R. Fisher and D.O.M., unpublished observations). Despite its inability to phosphorylate protein substrates, the CDK2 apoenzyme can bind ATP.

Crystallization, structure solution and refinement of both crystal structures are summarized in Table 1. The MIR electron density map calculated using the phases obtained from four heavy atom derivatives revealed most of the secondary structure elements of CDK2 (Fig. 1a). Multiple cycles of model building, refinement and phase combination yielded the final model, which includes all 298 residues in the sequence. A portion of the $2F_o - F_c$ density map is shown in Fig. 1b.

Backbone structure

The backbone structure of CDK2 can be divided into two lobes (Fig. 2a, b; topological diagram shown in Fig. 2d). The smaller N-terminal lobe of CDK2 consists of a sheet of five antiparallel β -strands ($\beta 1$ – $\beta 5$) and a single large helix ($\alpha 1$). The larger

‡ To whom correspondence should be addressed.

C-terminal lobe contains a pseudo-4-helical bundle ($\alpha 2, 3, 4, 6$), a small β -ribbon ($\beta 6$ – $\beta 8$), and two additional helices ($\alpha 5, 7$). The ATP-binding site is found in the cleft between the two lobes. The core (the β -sheet and the helical bundle) of the CDK2 structure is very similar to that of the cAPK catalytic subunit^{16–19}, even though these proteins are only distantly related at the amino-acid sequence level (about 24% identical). When the two kinase structures are optimally superimposed, about 44% of the C α atoms in CDK2 are within 1.5 Å of their position in cAPK. The C α atoms of the nine invariant residues found in all protein kinases⁵ are generally in very similar positions: all are within 0.9 Å except Glu51 (equivalent to Glu91 in cAPK; see later). This clearly argues that the catalytic cores of all eukaryotic protein kinases share considerable three-dimensional structural similarity, as predicted from primary structural homologies at key positions.

There are five major differences between the two protein kinase structures (Figs 2c and 3). First, the position and orientation of the α -helix of the N-terminal lobe, $\alpha 1$, are significantly different from those of the corresponding helix C in cAPK, and the small helix B in cAPK is absent in CDK2. Second, the helix in the L12 linker region of CDK2 (α L12) is absent in cAPK, which contains instead a short β strand (strand 9) in this region. The conformation of the loop following α L12 is also very different in the two proteins (some corresponding residues in this region are over 15 Å apart). Third, the orientation of $\alpha 5$ is different from that of the corresponding helix G in cAPK by 34°. Fourth, the L14 segment linking $\alpha 5$ and $\alpha 6$ is 25 residues longer in CDK2 and contains a small helix (α L14) that is not found in cAPK. Finally, the two proteins differ at their C termini, outside the most highly conserved kinase sequences: cAPK contains a pair of small helices (I and J) near its C terminus, whereas CDK2 has only a single helix ($\alpha 7$).

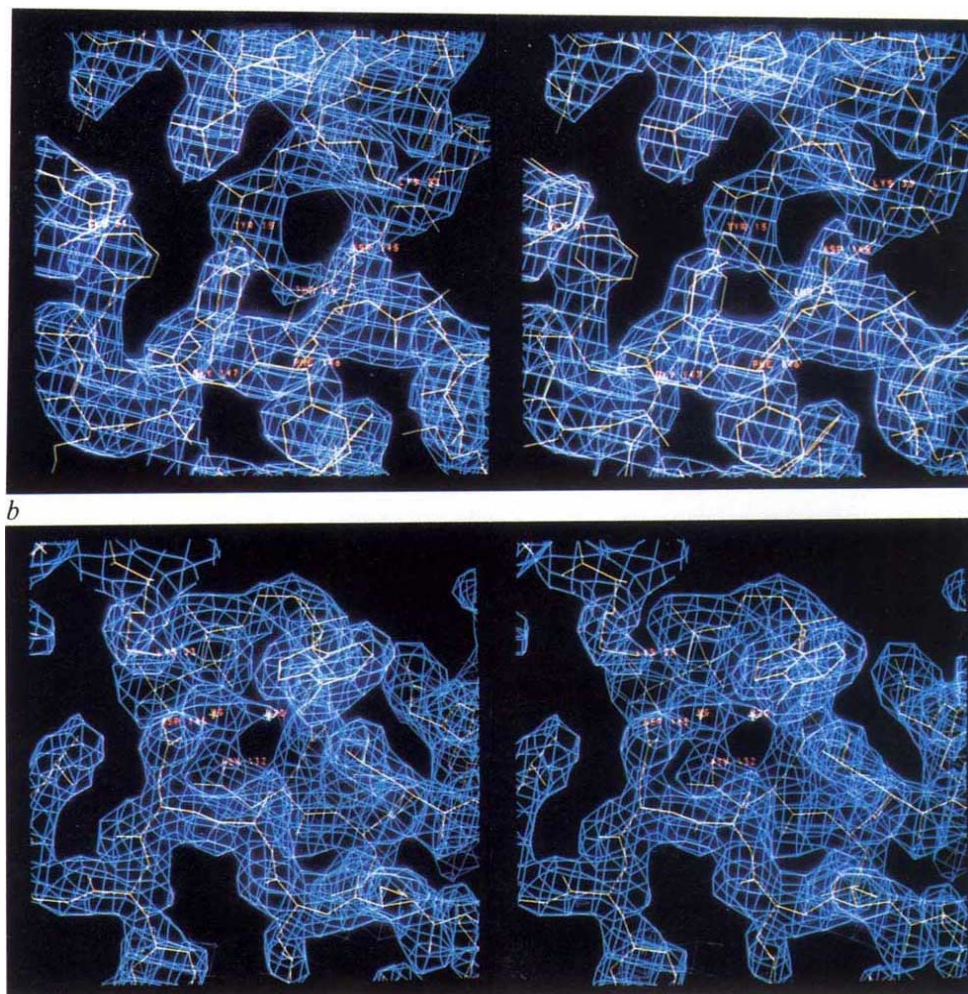
a

Catalytic sites in CDK2

The ATP-binding site. Our analysis of the CDK2–Mg²⁺ATP binary complex indicates that ATP interacts with several residues lining the cleft between the two lobes (Fig. 3b). The adenine base is positioned in a hydrophobic pocket between the β -sheet of the small lobe and the L7 loop between $\beta 5$ and $\alpha 2$. The ATP phosphates are held in position by ionic and hydrogen-bonding interactions with several residues, including Lys33, Asp145, and the backbone amides of the glycine-rich loop between $\beta 1$ and $\beta 2$ (Fig. 3b). An oxygen from each of the three phosphates of ATP contributes to the octahedral co-ordination of the Mg²⁺ ion (Fig. 3b). The three other ligands involved in the co-ordination of Mg²⁺ are Asp145, Asn132, and a water molecule. ATP binding appears to induce a slight closure of the cleft by a 2.1° hinge movement around an axis parallel to the longitudinal axis of the ATP molecule. Two highly conserved residues (Lys33 and Asn132) have significantly different orientations in the presence and absence of ATP (Fig. 3b).

Many of the ATP-binding-site interactions seen in CDK2 are similar to those in cAPK^{18, 19} (Fig. 3c), and several highly conserved residues in this region exhibit similar conformations in the two ATP-bound kinases (these residues include Asp127, Lys129, and Asn132 in the catalytic loop). There are also several important differences. First, there are differences in the conformations of Lys33 and Asp145, two critical residues involved in ATP phosphate binding (the equivalent residues in cAPK are Lys72 and Asp184, which are involved in phosphate orientation and metal ion coordination^{18, 19}). Second, several residues in the glycine-rich loop between $\beta 1$ and $\beta 2$ (particularly Thr14 and Tyr15) are significantly closer to the γ -phosphate of ATP. These differences are accompanied by major differences in the position of the Mg²⁺ ion and the conformation of the triphosphate

FIG. 1 a, Stereo view of the experimental solvent-flattened MIR electron density map after phase extension (from 3 Å to 2.7 Å resolution) showing the region containing Lys33, Glu51 and Asp145 in the CDK2 apoenzyme. The labelled residues are Thr14, Tyr15, Lys33, Glu51, Asp145, Phe146 and Gly147. b, Stereo view of the $2F_o - F_c$ electron density map showing the ATP-binding region of the Mg²⁺ATP–CDK2 binary complex. The labelled residues are Lys33, Asn132 and Asp145. The Mg²⁺ ion and one water molecule are also labelled.



group of ATP (Fig. 3c). The positions of the α - and γ -phosphates in CDK2 and cAPK are roughly similar, but the β -phosphates are about 4.5 Å apart. Owing to this difference in β -phosphate positions, there is a considerable difference in the alignment of the scissile bond between the β - and γ -phosphates with respect to the nucleophilic attacking group of the protein substrate (Fig. 3c and d).

These differences would be predicted to have significant effects on CDK2 activity. Based on studies of the kinase reaction mechanism in cAPK, it has been postulated that a general base in the catalytic loop (possibly Asp127 in CDK2) partially deprotonates the hydroxyl group of the serine in the protein substrate, catalysing nucleophilic attack by the hydroxyl oxygen on the γ -phosphate of ATP²¹. An inversion of the phosphorus configuration occurs during phosphotransfer, indicating that the γ -phosphate must be positioned in-line with the serine hydroxyl²². Indeed, in the cAPK ternary complex, the γ -phosphate of ATP is reasonably well aligned with the pseudo-substrate alanine side chain at the P-site of the inhibitor peptide^{18, 19} (Fig. 3c). However, in CDK2 the alignment of the

β - and γ -phosphates is almost perpendicular to the alignment in cAPK (Fig. 3c, d). Assuming that the serine substrate binds to CDK2 with roughly the same orientation as it does in cAPK, then in-line phosphotransfer does not appear to be possible in CDK2. This may provide some explanation for the inactivity of the CDK2 apoenzyme.

The protein substrate binding site. Based on the γ -phosphate position of ATP in the CDK2-Mg²⁺ATP complex, protein substrates presumably bind in the large cleft between the two lobes, allowing the substrate serine hydroxyl to be positioned near the γ -phosphate of ATP. Interestingly, the core of the protein substrate binding cleft in CDK2 is almost completely blocked by the large loop (the 'T-loop', residues 152–170, containing the Thr160 phosphorylation site) that follows the α L12 helix. Several residues in this loop block access to the γ -phosphate of ATP, and effective binding of the protein substrate to CDK2 would seem to be impossible. Thus, the T-loop appears to be acting as an auto-inhibitor of protein substrate binding. The quality of the electron density in this region of the CDK2 structure suggests that a portion of this loop

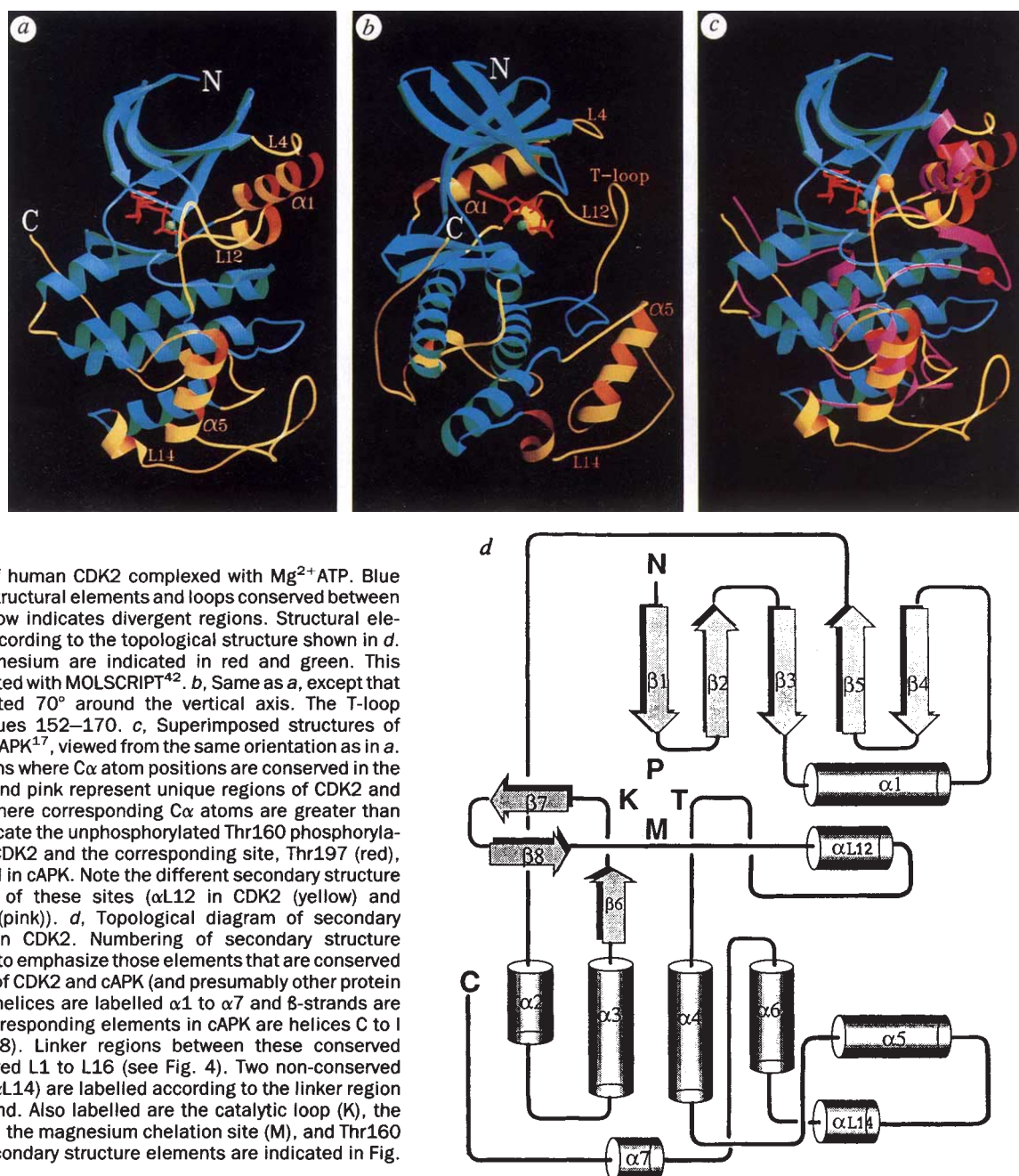


FIG. 2 *a*, Structure of human CDK2 complexed with Mg²⁺ATP. Blue indicates secondary structural elements and loops conserved between CDK2 and cAPK. Yellow indicates divergent regions. Structural elements are labelled according to the topological structure shown in *d*. Bound ATP and magnesium are indicated in red and green. This diagram was constructed with MOLSCRIPT⁴². *b*, Same as *a*, except that the structure is rotated 70° around the vertical axis. The T-loop corresponds to residues 152–170. *c*, Superimposed structures of CDK2-Mg²⁺ATP and cAPK¹⁷, viewed from the same orientation as in *a*. Blue represents regions where C α atom positions are conserved in the two kinases. Yellow and pink represent unique regions of CDK2 and cAPK, respectively, where corresponding C α atoms are greater than 1.5 Å apart. Balls indicate the unphosphorylated Thr160 phosphorylation site (orange) in CDK2 and the corresponding site, Thr197 (red), that is phosphorylated in cAPK. Note the different secondary structure elements N-terminal of these sites (α L12 in CDK2 (yellow) and β -strand 9 in cAPK (pink)). *d*, Topological diagram of secondary structure elements in CDK2. Numbering of secondary structure elements is designed to emphasize those elements that are conserved in the catalytic cores of CDK2 and cAPK (and presumably other protein kinases). Conserved helices are labelled α 1 to α 7 and β -strands are labelled β 1 to β 8 (corresponding elements in cAPK are helices C to I and β -strands 1 to 8). Linker regions between these conserved elements are numbered L1 to L16 (see Fig. 4). Two non-conserved elements (α L12 and α L14) are labelled according to the linker region in which they are found. Also labelled are the catalytic loop (K), the phosphate anchor (P), the magnesium chelation site (M), and Thr160 (T). Boundaries of secondary structure elements are indicated in Fig. 4.

(residues 156 to 162) is highly dynamic and possibly flexible in solution.

Regulatory sites in cyclin-dependent kinases

The high degree of primary sequence homology among members of the CDK family (55–65%) suggests that our human CDK2 structure will provide a useful framework for understanding all members of the family. Analysis of the location of highly conserved and solvent-accessible CDK residues should reveal those regions of the protein that interact with protein substrates, regulatory subunits, and the kinases and phosphatases that act on the CDK subunit. One hundred and forty residues are conserved among the four human and yeast CDKs aligned in Fig. 4. The side chains of 38 of these conserved residues are over 33% accessible to solvent, and therefore potential sites of interaction with other proteins (these residues are indicated by an 'S' beneath the alignment in Fig. 4). Conserved regions include the glycine-rich loop of the ATP binding site (between $\beta 1$ and $\beta 2$), the highly conserved PSTAIRE motif in the $\alpha 1$

helix, and the GDSEID motif at the N-terminal end of $\alpha 5$. The helix-loop segment containing the $\alpha L12$ helix and the T-loop (between Asp145 and Glu172) is also very highly conserved. Regions of considerable divergence can also be seen in the structure; these include the segment between $\alpha 2$ and $\alpha 3$, the large insert between $\alpha 5$ and $\alpha 6$, and the C-terminal tail. These divergent segments tend to be found in areas that are relatively distant from the active site.

Phosphorylation sites. The inhibitory phosphorylation sites Thr14 and Tyr15 are both in the middle of the glycine-rich loop that serves as a phosphate anchor in ATP binding. These residues are unphosphorylated in our structure, and the side chains of both residues are inaccessible to solvent owing to the presence of the T-loop that follows $\alpha L12$. The hydroxyl of Thr14 is very close to the γ -phosphate of ATP, and phosphorylation here would directly disrupt the conformation of the ATP phosphates. The hydroxyl of Tyr15, on the other hand, is more distant from ATP. It is not clear that a phosphate at this site would directly interfere with ATP conformation (as suggested

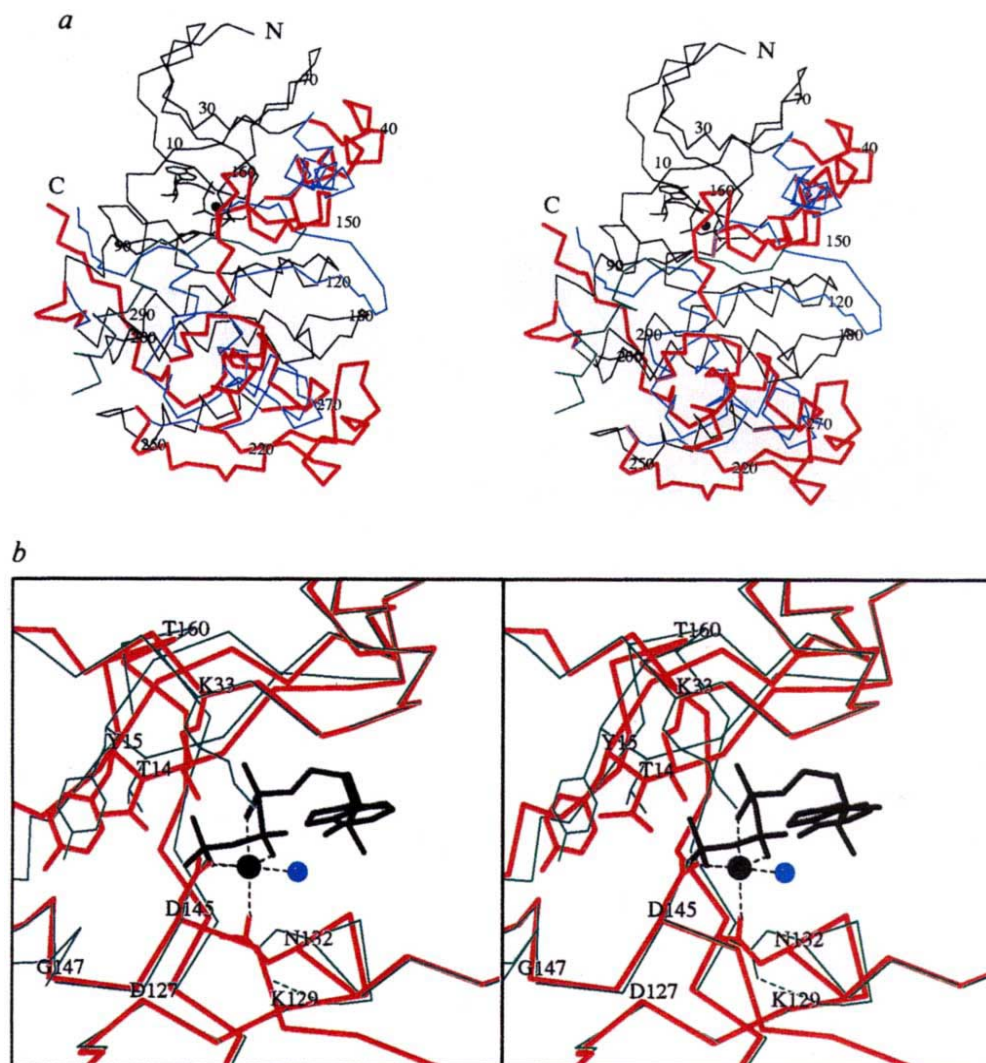


FIG. 3 *a*, Stereo view of the superimposed C α traces of the binary CDK2-Mg²⁺ATP complex and the binary cAPK-peptide inhibitor complex¹⁷. Coloured lines represent the following structures. Thin black, regions of CDK2 that are structurally similar in the two kinases (as in Fig. 2*a-c*); bold red, structurally unique regions of CDK2 (as in Fig. 2*a-c*); thin blue, structurally unique regions of cAPK; thin green,

peptide inhibitor in cAPK structure. Mg²⁺ATP in the CDK2 structure is also shown in black. Residue numbers refer to CDK2. *b*, Stereo view showing the main differences between the structures of CDK2 (thin green) and its Mg²⁺ATP complex (bold red). The C α traces are shown, as well as side chains with significantly different conformations and those involved in co-ordination of Mg²⁺. In order to avoid bias from the

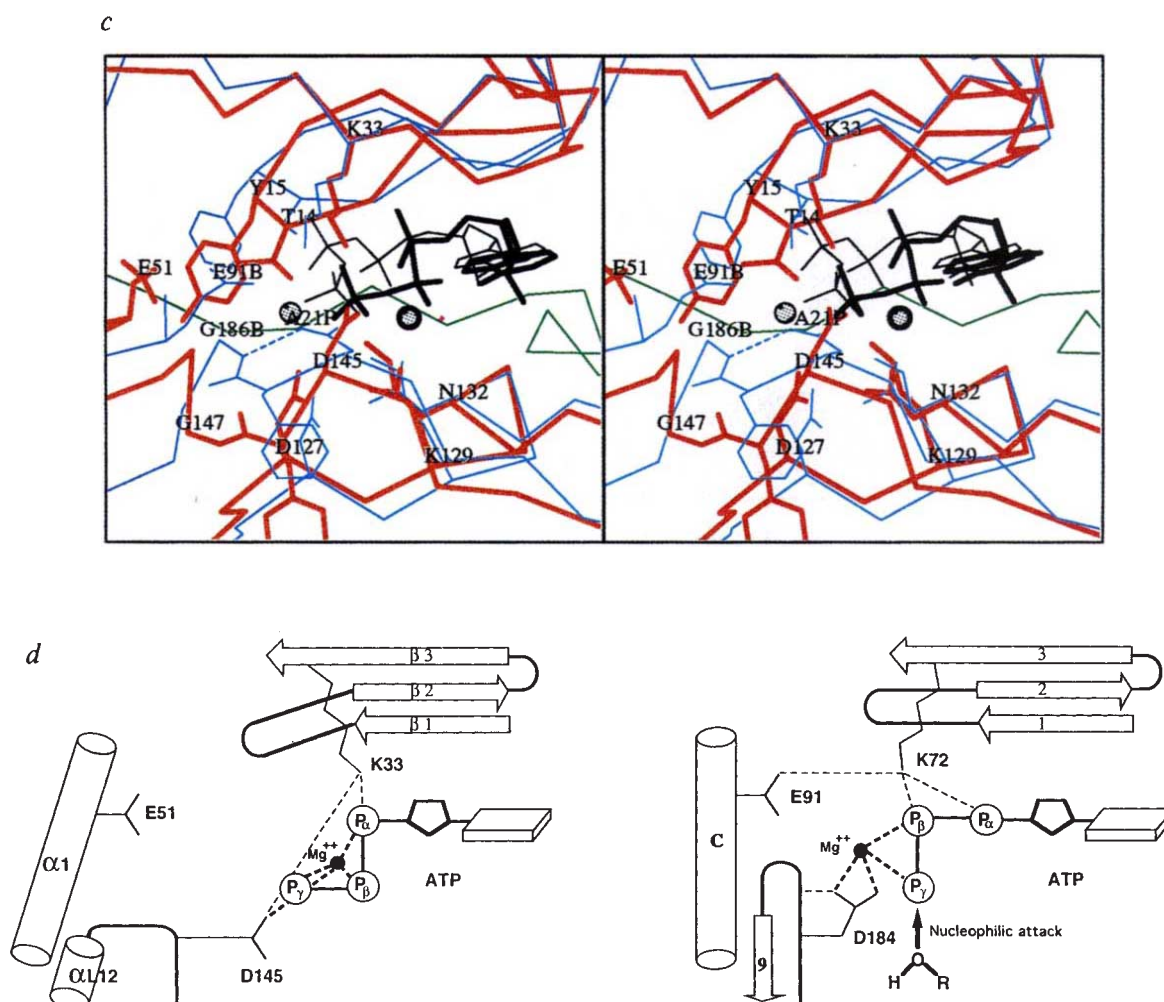
by recent work showing that Tyr15 phosphorylation has little effect on ATP binding²³. Phosphorylation at one or both of these sites might also inhibit kinase activity by interfering with protein substrate binding or by indirectly affecting ATP orientation by changing the conformation of the glycine-rich loop.

The activating phosphorylation site, Thr160, is found at the apex of the T-loop blocking the protein substrate-binding site. The hydroxyl group of Thr160 is oriented toward the glycine-rich loop of the ATP-binding site, and although Thr160 as a whole is more than 33% exposed to solvent, its hydroxyl group appears to be inaccessible. The electron density map in this area is not of sufficient quality to allow a detailed analysis of atomic interactions.

The cyclin-binding site. Previous studies of mutant CDC2 proteins allow some speculation about the regions of CDK2 involved in binding the cyclin regulatory subunit. Charged-to-alanine scanning mutagenesis of human CDC2, whereby small groups of charged residues are changed to alanine, has shown that cyclin A binding is inhibited by mutations in several clusters

of residues in the N-terminal lobe²⁴. In the CDK2 tertiary structure, seven residues implicated in these studies (Glu8, Lys9, Glu12, Arg36, Asp38, Glu40, Glu42) are more than 33% accessible to solvent and are probably exposed on the protein surface. These residues are found in adjacent regions: one at the C-terminal end of β 1 and the other in the loop between β 3 and α 1 (Asp38, Glu40 and Glu42 form a concentrated acidic patch on the surface of this loop; Fig. 5).

The CKS-binding site. CDKs can also interact with other potential regulatory subunits known in human cells as the CKS proteins (known as p13^{suc1} in *S. pombe*)²⁵. These proteins interact tightly with CDKs *in vivo* and *in vitro*, but their function is unclear. Charged-to-alanine mutagenesis of human CDC2 has identified several clusters of charged residues where mutation inhibits CKS binding²⁶. These sites are scattered in the tertiary structure: two clusters are in the N-terminal lobe (equivalent to Lys20–Lys24 and Lys56–His60), while four clusters are found in a limited region of the C-terminal lobe (equivalent to Arg 169–Glu172, Tyr179–Asp185, Arg214–Arg217, and Glu270–Arg274).



CDK2 apoenzyme structure, all regions that interact with ATP in the ATP complex structure have been rebuilt in the omitmap electron density calculated after simulated annealing refinement without ATP and surrounding residues. A difference Fourier synthesis revealed a Mg²⁺ ion (black ball), which was octahedrally co-ordinated with 6 ligands: 3 oxygens, one from each phosphate, a carboxyl oxygen of Asp145, the oxygen of the Asn132 sidechain, and an oxygen of a water molecule (blue ball). c, Close-up of superimposed ATP-binding sites in the binary CDK2–Mg²⁺ATP complex and the ternary cAPK–Mn²⁺ATP – peptide inhibitor complex¹⁹. Coloured lines represent the following structures.

Bold red, CDK2; bold black, Mg²⁺ATP in CDK2; thin blue, cAPK; thin black, Mn²⁺ATP in cAPK (the minor metal ion site¹⁹ is not shown); thin green, peptide inhibitor in cAPK. Numbers refer to residues in CDK2, except those numbers followed by 'B' (which refer to residues in cAPK) and those followed by 'P' (which refer to residues in the peptide inhibitor). The T loop (residues 152–170) in CDK2 is not shown for clarity. d, Schematic drawing of ATP-binding pocket in CDK2 (left) and cAPK (right). α -Helices are shown as cylinders and β -strands as arrows. Potential hydrogen bonds are shown as thin dashed lines and co-ordination bonds as thick dashed lines.

About 20 point mutations have been identified that confer a temperature-sensitive defect on yeast CDK proteins^{27–30}. Some of these mutants (for example, *cdc2-33*, 56, L7, 45 and 17 in *S. pombe*) can be rescued by overexpression of the CKS protein, suggesting a defect in CKS binding. The equivalent residues in CDK2 (170, 176, 201, 203 and 205) are found in a small region of the C-terminal lobe and have low solvent-accessibility. Some of these mutant proteins (Cdc2-33, 56, L7) have temperature-sensitive kinase activity *in vitro*^{31, 32}, suggesting that the residues affected in these mutants are important in protein conformation.

Regulation and conformational switching

The previously reported structure of cAPK represents an active enzyme conformation, whereas our CDK2 structure is that of an

inactive kinase. We therefore assume that differences between the active sites of cAPK and CDK2 reveal regions that are responsible for the inactivity of the CDK2 apoenzyme. A comparison of the two kinases suggests that the low kinase activity of CDK2 is due to two major structural constraints. First, critical side chains in the ATP-binding site (mainly Lys33 and Asp145), as well as the β -phosphate of ATP, are positioned in CDK2 in such a way that phosphotransfer is inhibited. Second, the T-loop in the L12 linker region blocks the protein substrate-binding cleft.

The different conformations of the ATP-binding pockets in CDK2 and cAPK are probably due to differences in nearby secondary structure elements (Fig. 3d). The ATP-binding region of CDK2 is adjacent to a short helix (α L12), instead of the β -strand that exists in the same region of cAPK. Owing to

TABLE 1 Diffraction data, structure solution and refinement statistics

Diffraction data

	Native	Pt-1*	Hg-1†	Pb-1‡	Hg-2§
Resolution (Å)	1.9	2.7	2.1	2.1	2.1
Total observations ($I > 3\sigma(I)$)	78,176	25,981	49,683	55,096	53,082
Unique reflections	20,634	8,001	16,507	17,037	16,924
Completeness of data (%)	85.4	92.5	90.5	93.3	89.8
R_{sym} (%)	6.5	5.6	6.3	4.6	6.9

Structure solution statistics

Derivative	Resolution (Å)	Unique reflections	R_{iso} (%)¶	Number of sites	R_c (%)#	Figure of merit	Phasing power**
Pt-1*	3.0	5,052	10.7	1	57.4	0.27	1.09
Hg-1†	3.0	5,378	15.5	2	49.7	0.41	2.20
Pb-1‡	3.0	5,506	16.1	2	52.6	0.31	1.48
Hg-2§	3.0	5,449	16.0	2	50.0	0.39	1.94

Refinement statistics

	R value (%)	Resolution (Å)	Number of reflections $F > 2\sigma(F)$	R.m.s. deviation	
				Bond lengths (Å)	Bond angles (°)
CDK2(APO)	18.5	8.0–2.4	10,945	0.016	3.5
CDK2(Mg ²⁺ ATP)	18.4	8.0–2.4	10,632	0.016	3.5

Crystallization of human CDK2 has been described²⁰. Briefly, a CDK2 solution was concentrated to 10 mg ml⁻¹ by dialysis against 20 mM HEPES buffer (pH 7.4) and 1 mM EDTA. Sitting drops were equilibrated by vapour diffusion at 4°C against reservoirs containing 200 mM HEPES buffer, pH 7.4. Diamond and wedge-shaped crystals appeared after 2 to 4 days. A gradual increase of the HEPES concentration in the reservoirs (up to 800 mM) produced crystals with average dimensions of about 0.2 mm × 0.3 mm × 0.3 mm. The crystals diffract up to 1.9 Å on the RAXIS II imaging plate using a RIGAKU rotating anode X-ray generator at a loading of 50 kV, 100 mA. Crystals are orthorhombic and belong to the space group $P2_12_12_1$, with unit cell dimensions $a = 73.12$ Å, $b = 72.72$ Å and $c = 54.25$ Å. They have a calculated packing density³⁶ (V_m) of 2.126 Å³ per dalton (1 molecule in the asymmetric unit). The Mg²⁺ATP-CDK2 complex was obtained by soaking the crystals in a 50mM HEPES solution containing 2.5 mM MgCl₂ and 1.25 mM ATP. Four isomorphous heavy-atom derivatives were prepared by soaking CDK2 apoenzyme crystals in a 50mM HEPES solution (pH 7.4) in which the heavy metal compound was dissolved. Heavy-atom sites were solved from difference Pattersons and difference Fouriers. Two different sites (one close to Cys 118 and the other close to Cys 177) were occupied to different extents in each of the four crystal soaks. Multiple isomorphous replacement (MIR) refinement statistics are given. The MIR phases, with an overall figure of merit of 0.64 (5,702 reflections between 30 and 3.0 Å) yielded an excellent electron density map with many interpretable features. The overall interpretability of the map was improved with solvent-flattening, using automatic boundary definition^{37, 38} and phase extension to 2.7 Å. Much of the overall backbone of the molecule could be traced in the solvent-flattened map (for example, see Fig. 1a) using the 'O' modelling program³⁹ on a Silicon Graphics Personal Iris. At this stage, some of the connections between elements of well-defined secondary structure were cross-checked with the published cAPK backbone¹⁶. This initial model was refined by simulated annealing with native data to 2.7 Å using the program package XPLOR⁴⁰. Phase combination between the model and solvent-flattened MIR phases was carried out, and new maps to 2.7 Å resolution were computed. Most polypeptide connections could then be traced unambiguously. Several additional cycles of model building, refinement, and phase combination were done using native data to 2.4 Å. The current molecular model includes all 298 residues in the protein sequence, as well as 41 water molecules. Individual temperature factors were refined in the final cycle. High B factors (greater than 50 Å²) are found in the following loop regions: L2 (residues 11–15), L3 (25–26), L4 (36–46), L6 (72–75), the T-loop (153–161) and the C terminus (295–298). Two residues violate the Ramachandran plot (Arg 36 and Leu 37). A portion of the $2F_o - F_c$ map is shown in Fig. 1b.

* Pt-1, *cis*-platinum(II)diamine chloride (1.2 mg ml⁻¹, 18 h).

† Hg-1, sodium salt of ethylmercury thiosalicylic acid (0.75 mg ml⁻¹, 4 h).

‡ Pb-1, trimethyllead acetate (0.5 mg ml⁻¹, 2d).

§ Hg-2, ethylmercury phosphate (0.05 mg ml⁻¹, 2d)⁴¹.

¶ R_{sym} (%) = $100 \sum_i |I_i| / \sum_i I_i$ and $\langle I_i \rangle$ is the average of I_i over all symmetry equivalents.

¶ R_{iso} (%) = $100 \sum_i |F_{\text{PH}}^2 - F_{\text{P}}^2| / \sum_i (F_{\text{PH}}^2 + F_{\text{P}}^2)$ where F_{PH} and F_{P} are the derivative and native structure factors, respectively.

R_c (%) = $100 \sum_i |F_{\text{PH}} \pm F_{\text{P}}| - |F_{\text{H}}(\text{calc})| / \sum_i |F_{\text{PH}} \pm F_{\text{P}}|$ for centric reflections.

** Phasing power = $\langle F_{\text{H}} \rangle / E$; the r.m.s. heavy-atom structure factor amplitude divided by the residual lack of closure error.

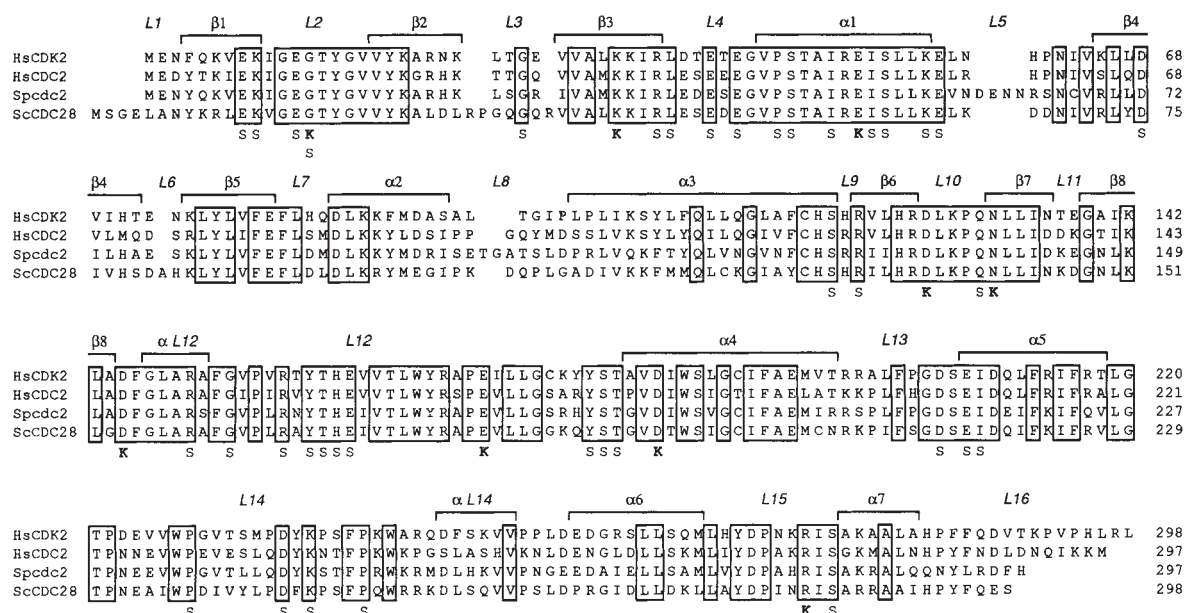


FIG. 4 Alignment of human and yeast cyclin-dependent kinase sequences (HsCDK2, human CDK2⁴³; HsCDC2, human CDC2⁴⁴; Spcdc2, *S. pombe* CDC2⁴⁵; ScCDC28, *S. cerevisiae* CDC28⁴⁶). Boxes surround residues conserved in all four kinases. Secondary structure elements in human CDK2, numbered as in Fig. 2d, are indicated above the alignment. Linker regions between conserved structural elements

are labelled L1 to L16. α -Helical segments within linker regions L12 and L14 are labelled α L12 and α L14, respectively. Nine residues found in almost all protein kinases⁵ are indicated by a 'K' below the alignment. 'S' indicates solvent-accessible residues that are conserved in all four CDKs. These residues have a solvent-accessible surface⁴⁷ that is at least 33% of the surface area of the fully exposed residue⁴⁸.

the presence of α L12, the adjacent helix α 1 is positioned farther away from ATP, corresponding to a 24° rotation around its C-terminal end (Figs 2c and 3d). In cAPK, a glutamate residue in the middle of this helix (Glu91) interacts with Lys72. The corresponding interaction (Glu51/Lys33) is not possible in CDK2, presumably explaining the altered position of Lys33 (Fig. 3c, d). In addition, the α L12 helix imposes constraints on the positions of three conserved residues immediately N-terminal of the helix: Asp145, Phe146 and Gly147 (the DFG motif). In cAPK, these residues form a tight loop held together by hydrogen-bonding between the α -carbonyl of the aspartate

(Asp184) and the backbone amide of the glycine (Gly186)^{16, 17}. In CDK2 this contact is prevented by the helical conformation of α L12, resulting in the different orientation of the Asp145 side chain. This side chain also has an altered position as a result of interactions with the differently positioned Lys33 side chain (Fig. 3c, d).

The altered ATP orientation in CDK2 may also be due to the different position of the glycine-rich loop of the phosphate anchor (Fig. 3c, d). Relative to cAPK, this loop in CDK2 is positioned closer to ATP, perhaps destabilizing the active β -phosphate conformation that is seen in cAPK. Residues of the

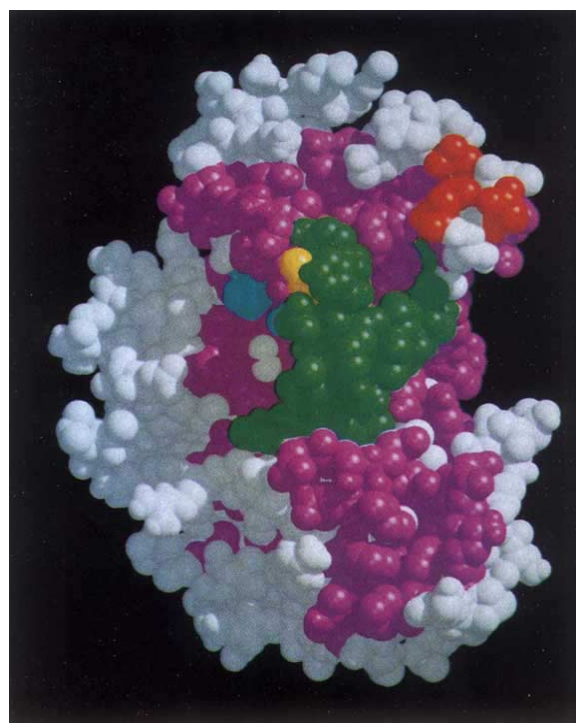


FIG. 5 Space-filling model of human CDK2, viewed along the same direction as that in Fig. 2a and c. Pink indicates residues conserved in the four CDK sequences shown in Fig. 4; this side of the molecule shows the largest number of conserved residues. Blue indicates the ATP molecule. Red indicates a cluster of three acidic residues (Asp38, Glu40, Glu42) that has been implicated in cyclin binding (see text). For size comparison, note that typical cyclins (such as cyclin A, B and E) contain about 400 amino acids; a conserved 100-residue region in the middle of the cyclin sequence is thought to be involved in CDK binding and activation^{49, 50}. The T loop (residues 152-170) is indicated in green and Thr160 is indicated in yellow.

T-loop are close to the glycine-rich loop and may also contribute to its altered position.

The initial step in CDK2 activation is thought to be cyclin binding, which yields a CDK2-cyclin complex with low kinase activity. Phosphorylation of the cyclin-bound CDK2 at Thr160 leads to complete activation^{7,8,10}. These processes may activate CDK2 by inducing conformational changes that remove the structural constraints imposed by the helix-loop segment that contains α L12 and the T-loop. If cyclin binds to the region of the N-terminal lobe above the cleft, as suggested by mutagenesis experiments (Fig. 5)²⁴, then it will clearly be in an ideal position to affect the conformation of these structures. For example, cyclin binding might cause α L12 to lose its helical character, allowing the residues of the ATP-binding site to achieve the active conformations found in cAPK. The 'melting' of α L12 would also increase the flexibility of the T-loop, allowing this loop to be re-oriented away from the substrate-binding site.

Cyclin-induced changes in the flexibility or position of the T-loop might explain previous observations that phosphorylation at Thr160, Thr14 and Tyr15 is cyclin-dependent *in vitro*⁷⁻⁹. The hydroxyl groups of these residues are buried in our CDK2 structures but would be uncovered if the T-loop were removed by cyclin binding. On the other hand, these sites may be partially accessible in solution, because the upper portion of the T-loop appears to be flexible in our structure. If this is the case, then the cyclin-dependence of phosphorylation might suggest that cyclin also forms part of the substrate recognition site for the kinases that act on these residues.

Thr160 phosphorylation could contribute to the activation process by stabilizing the T-loop in an active conformation on the C-terminal lobe in a position similar to that found in cAPK, where this loop is stabilized by ionic interactions between an

autophosphorylated threonine (Thr197) and several cationic residues (His87, Arg165, and Lys189)¹⁶. Basic residues are found at two of these positions in CDK2 (Arg126 and Arg150). Thus, a re-positioned T-loop could be stabilized by interactions between these residues and the Thr160 phosphate. Stabilization of the T-loop by phosphorylation may also stabilize or improve the cyclin-binding site. CDC2 binding to cyclin A is inhibited by mutation of this phosphorylation site²⁴, although the binding of several other complexes (CDC2-cyclin B, CDK2-cyclin A or B) is not affected^{7,9,23} (D. Desai and D.O.M., unpublished observations).

Phosphorylation of residues in this region appears to be a mechanism of activation in many protein kinases. Members of the protein-serine/threonine kinase subfamily (cAPK, CDK and the MAP/ERK kinases) as well as the protein-tyrosine kinase subfamily (c-Src and the insulin receptor) are activated by phosphorylation or autophosphorylation at residues in this segment between the conserved 'DFG' and 'APE' motifs (Asp145 and Glu172 in CDK2)³³⁻³⁵. All of these kinases also contain basic residues at positions equivalent to Arg165 and Arg189 in cAPK (Arg126 and Arg150 in CDK2). Thus, cationic pockets may exist in these kinases for binding the phosphorylated regulatory sites and stabilizing an active enzyme conformation.

The cyclin-dependent protein kinases are a unique group of enzymes whose catalytic subunits are controlled by a complex array of regulatory inputs. Our studies suggest that the CDK regulatory mechanism involves a helix-loop region adjacent to the active site of the enzyme. A complete understanding of CDK regulation will require additional analyses of CDK2 in the presence of cyclin and Thr160 phosphorylation. □

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- Norbury, C. & Nurse, P. A. *Rev. Biochem.* **61**, 441-470 (1992).
- Forsburg, S. L. & Nurse, P. A. *Rev. Cell Biol.* **7**, 227-256 (1991).
- Fang, F. & Newport, J. W. *Cell* **66**, 731-742 (1991).
- Pagano, M. et al. *J. Cell Biol.* **121**, 101-111 (1993).
- Hanks, S. K. & Quinn, A. M. *Meth. Enzym.* **200**, 38-62 (1991).
- Hunt, T. *Curr. Opin. Cell Biol.* **1**, 286-274 (1989).
- Desai, D., Gu, Y. & Morgan, D. O. *Molec. Biol. Cell* **3**, 571-582 (1992).
- Connell-Crowley, L., Solomon, M. J., Wei, N. & Harper, J. W. *Molec. Biol. Cell* **4**, 79-92 (1993).
- Solomon, M. J., Lee, T. & Kirschner, M. W. *Molec. Biol. Cell* **3**, 13-27 (1992).
- Gu, Y., Rosenblatt, J. & Morgan, D. O. *EMBO J.* **11**, 3995-4005 (1992).
- Gould, K. L., Moreno, S., Owen, D. J., Sazer, S. & Nurse, P. *EMBO J.* **10**, 3297-3309 (1991).
- Krek, W. & Nigg, E. A. *EMBO J.* **10**, 3331-3341 (1991).
- Norbury, C., Blow, J. & Nurse, P. *EMBO J.* **10**, 3321-3329 (1991).
- Parker, L. L., Atherton-Fessler, S. & Piwnicka-Worms, H. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2917-2921 (1992).
- McGowan, C. H. & Russell, P. *EMBO J.* **12**, 75-85 (1993).
- Knighton, D. R. et al. *Science* **253**, 414-420 (1991).
- Knighton, D. R. et al. *Science* **253**, 407-414 (1991).
- Zheng, J. et al. *Biochemistry* **32**, 2154-2161 (1993).
- Zheng, J. et al. *Acta crystallogr.* **D49**, 363-365 (1993).
- Rosenblatt, J., De Bondt, H., Jancarik, J., Morgan, D. O. & Kim, S.-H. *J. molec. Biol.* **230**, 1317-1319 (1993).
- Yoon, M.-Y. & Cook, P. F. *Biochemistry* **26**, 4118-4125 (1987).
- Ho, M.-F., Bramson, H. N., Hansen, D. E., Knowles, J. R. & Kaiser, E. T. *J. Am. Chem. Soc.* **110**, 2680-2681 (1988).
- Atherton-Fessler, S., Parker, L. L., Geahlen, R. L. & Piwnicka-Worms, H. *Molec. cell Biol.* **13**, 1675-1685 (1993).
- Ducommun, B. et al. *EMBO J.* **10**, 3311-3319 (1991).
- Richardson, H. E., Stueland, C. S., Thomas, J., Russell, P. & Reed, S. I. *Genes Dev.* **4**, 1332-1344 (1990).
- Ducommun, B., Brambilla, P. & Draetta, G. *Molec. cell Biol.* **11**, 6177-6184 (1991).
- Ayscough, K., Hayles, J., MacNeill, S. A. & Nurse, P. *Molec. gen. Genet.* **232**, 344-350 (1992).

- Carr, A. M., MacNeill, S. A., Hayles, J. & Nurse, P. *Molec. gen. Genet.* **218**, 41-49 (1989).
- Lorincz, A. T. & Reed, S. I. *Molec. cell Biol.* **6**, 4099-4103 (1986).
- MacNeill, S. A., Creanor, J. & Nurse, P. *Molec. gen. Genet.* **229**, 109-118 (1991).
- Boother, R. N., Alfa, C. E., Hyams, J. S. & Beach, D. H. *Cell* **58**, 485-497 (1989).
- Moreno, S., Hayles, J. & Nurse, P. *Cell* **58**, 361-372 (1989).
- Payne, D. M. et al. *EMBO J.* **10**, 885-892 (1991).
- Kmieciak, T., Johnson, P. J. & Shalloway, D. *Molec. cell Biol.* **8**, 4541-4546 (1988).
- Ellis, L. et al. *Cell* **45**, 721-732 (1986).
- Matthews, B. W. *J. molec. Biol.* **33**, 491-497 (1968).
- Furey, W. *PHASES Manual* (Univ. Pittsburgh, PA, 1992).
- Wang, B. C. *Meth. Enzym.* **115**, 90-112 (1985).
- Jones, T. A., Zou, J.-Y., Cowan, S. W. & Kjeldgaard, N. *Acta crystallogr.* **A47**, 110-119 (1991).
- Brunker, A. T. *XPLOR, a System for Crystallography and NMR, Version 3.0 Manual* (Yale Univ., New Haven, CT, 1991).
- Petsko, G. A. *Meth. Enzym.* **114**, 147-156 (1985).
- Kraulis, P. J. *J. appl. crystallogr.* **24**, 946-950 (1991).
- Tsai, L.-H., Harlow, E. & Meyerson, M. *Nature* **353**, 174-177 (1991).
- Lee, M. G. & Nurse, P. *Nature* **327**, 31-35 (1987).
- Hindley, J. & Phear, G. A. *Gene* **31**, 129-134 (1984).
- Lorincz, A. T. & Reed, S. I. *Nature* **307**, 183-185 (1984).
- Kabsch, W. & Sander, C. *Biopolymers* **22**, 2577-2637 (1983).
- Rose, G. D., Geselowitz, A. R., Lesser, G. J., Lee, R. H. & Zehfus, M. H. *Science* **229**, 834-838 (1985).
- Kobayashi, H. et al. *Molec. Biol. Cell* **3**, 1279-1294 (1992).
- Lees, E. M. & Harlow, E. *Molec. cell Biol.* **13**, 1194-1201 (1993).

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Single-shell carbon nanotubes of 1-nm diameter

Sumio Iijima & Toshinari Ichihashi

Fundamental Research Laboratories, NEC Corporation,
34 Miyukigaoka, Tsukuba, Ibaraki 305, Japan

CARBON nanotubes¹ are expected to have a wide variety of interesting properties. Capillarity in open tubes has already been demonstrated²⁻⁵, while predictions regarding their electronic structure⁶⁻⁸ and mechanical strength⁹ remain to be tested. To examine the properties of these structures, one needs tubes with well defined morphologies, length, thickness and a number of concentric shells; but the normal carbon-arc synthesis^{10,11} yields a range of tube types. In particular, most calculations have been concerned with single-shell tubes, whereas the carbon-arc synthesis produces almost entirely multi-shell tubes. Here we report the synthesis of abundant single-shell tubes with diameters of about one nanometre. Whereas the multi-shell nanotubes are formed on the carbon cathode, these single-shell tubes grow in the gas phase. Electron diffraction from a single tube allows us to confirm the helical arrangement of carbon hexagons deduced previously for multi-shell tubes¹.

We found the single-shell tubules in soot-like deposits formed in a carbon-arc chamber similar to that used for fullerene production. Two vertical electrodes are installed in the centre of the chamber. The anode, which is the upper electrode, is a graphitic carbon rod 10 mm in diameter, and the cathode, a 20-mm-diameter carbon rod, has a shallow dimple used to hold a small piece of iron during evaporation. The evaporation chamber is filled with a gas mixture typically consisting of 10 torr methane and 40 torr argon. The carbon discharge arc was generated by running a d.c. current of 200 A at 20 V between the electrodes. The iron filings melted to form a droplet, and so generated iron vapour which cooled and condensed into small particles of iron carbide above the cathode. The vaporization of iron takes place simultaneously with the production of soot, both from methane and by evaporation from the carbon cathode. We noticed that no tubules were formed when the carbon arc reactor was operated with any one of the three components argon, iron and methane absent.

The catalytic role of iron is well known in carbon fibre production, in which iron particles are found on the fibre tips and act as heterogeneous deposition centres for carbon atoms from the vapour phase¹². In the present experiment, we did not find iron particles at the tubule tips. We suspect, however, that atomic iron particles, presumably as a homogeneous catalyst in the vapour phase, somehow assist the formation of single-shell tubules. Specimens for electron microscopy were prepared from acetone suspensions of soot collected over the electrodes in the evaporation chamber. Nanotubes were placed on a microscope specimen grid by drying a drop of the suspension taken up on the grid. We used either an ultra-high-vacuum (JEM 200FXV) or conventional transmission electron microscope (Topcon 002B) at 120 kV or 200 kV accelerating voltage.

A typical electron micrograph showing a general view of the specimen is reproduced in Fig. 1a. Threads are curved and tangled together to form bundles in some regions. All the threads are found to be single-shell carbon nanotubes. Round, dark objects attached to the tubules are cementite (Fe_3C), as indicated by powder diffraction measurements and measurement of the d spacings of lattice fringes (lattice images with spacings of around 0.2 nm were recorded on individual particles); these particles range in size from a few nanometres to several tens of nanometres, which were mostly coated with a few graphite layers. Some of the particles were soluble in nitric acid.

The nanotubes often form as bundles, but isolated and single tubules are also present. Three tubules which bridge two cementite particle agglomerates are seen in Fig. 1b. On the upper right-hand side, a graphitic layer showing an image of the basal plane with lattice spacing 0.34 nm can be recognized. From this, we calibrated the magnifications of the tubules. All micrographs were recorded at optimum focus¹³, so that two dark lines in the tubule image correspond to side portions of the cylinders. The thinnest tube in this micrograph (labelled 1) is 0.75 nm in diameter, and is attached to a thicker (13-nm) tube (labelled 2). Tubules 1 and 2 are slightly curved, but tubule 3 (diameter 0.92 nm) spans straight across a 140-nm opening between two cementite particle agglomerates. The longest single-shell tubule was 700 nm long with a diameter of 0.9 nm.

Short and terminated tubules are commonly observed (4 and 5 in Fig. 1b). The terminated tubule labelled 6 moved gradually during the observation so that one end appears to be fading out. No cementite particles were found on the free tips of the tubules, but they are entangled with cementite particle agglomerates. The tubules are capped in shapes that have been reported previously^{14,15} (arrow 4 in Fig. 2).

We carefully measured the diameters on the electron micrographs of individual tubules. Figure 2 shows a histogram of the diameters of about 60 tubules, which range from about 0.7 nm to 1.6 nm. Two peaks are seen at tubule diameters of around 0.8 nm and 1.05 nm; we believe that these peaks are significant. The origin of the preferred tubule diameters is interesting to consider in terms of the tubule helicity and growth.

Figure 3a shows an electron diffraction pattern taken from a 1.37-nm single-shell tubule together with its corresponding

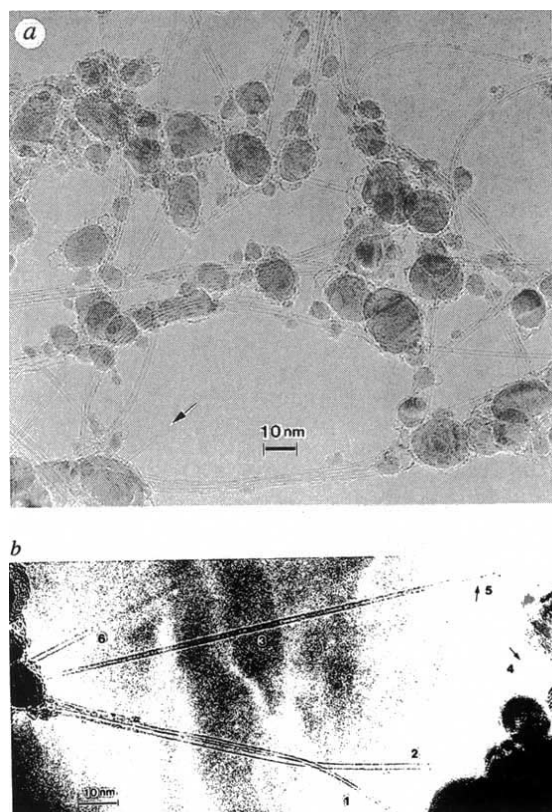


FIG. 1a, Electron micrograph showing bundles of single-shell carbon nanotubes which are curved and entangled. Dark blobs are cementite particles which assist in tubule growth. A terminated tubule is indicated by an arrow. b, Electron micrograph showing individual single-shell nanotubes. The tubule labelled 1 is 0.75 nm in diameter and tubule 2 is 1.37 nm in diameter. A straight tubule (3) and two terminated ones (4 and 5) can also be seen.

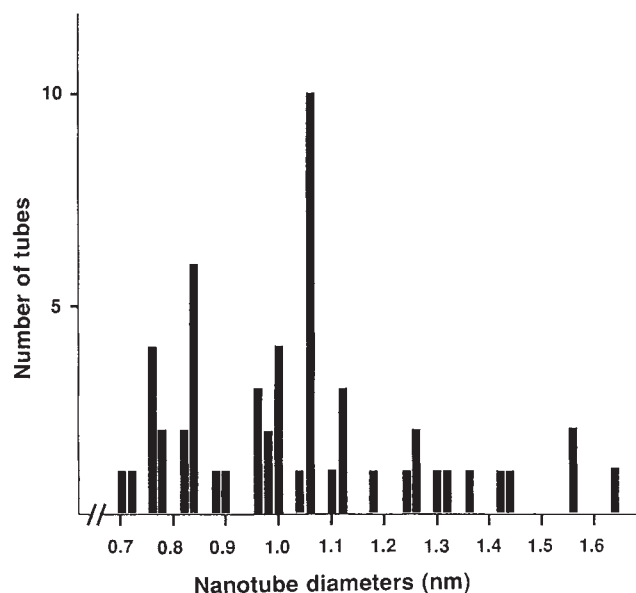


FIG. 2 Histogram showing frequency of single-shell nanotubes of different tubule diameters. Two peaks, at 0.8 nm and 1.35 nm, are dominant.

electron micrograph (Fig. 3b). The tubule is attached to the bundle of tubules seen at the left-hand side. An electron beam (20 nm diameter) was focused on to the tubule, so that the area diffracting the electrons comprised about 2,000 carbon atoms. This small scattering volume and the cylindrical structure are responsible for the extremely weak and diffused diffraction intensities. There are two hexagonal ($hk0$) diffraction patterns rotated $\pm \alpha/2$ from perfect alignment along the tube axis, and these patterns show mirror symmetry both along and perpendicular to the tube axis. The mirror symmetry confirms helicity in the single-shell tubule structure, as was found previously for

multi-shell tubules¹. All electron diffraction patterns observed in the present sample showed the $2mm$ symmetry. The $(00l)$ spots coming from the graphite basal lattice plane, which were observed for the multi-shell tubes, are completely absent (see Fig. 3 of ref. 1).

The spots are streaked vertically because of the fibrous structure. Each streaked spot in Fig. 3a has intensity maxima appearing with a period (indicated by triple arrows) of 0.73 nm^{-1} . The value corresponds to the diameter measured on the tubule (1.37 nm). The modulation can be explained by Fraunhofer diffraction from two vertical portions of the tubule

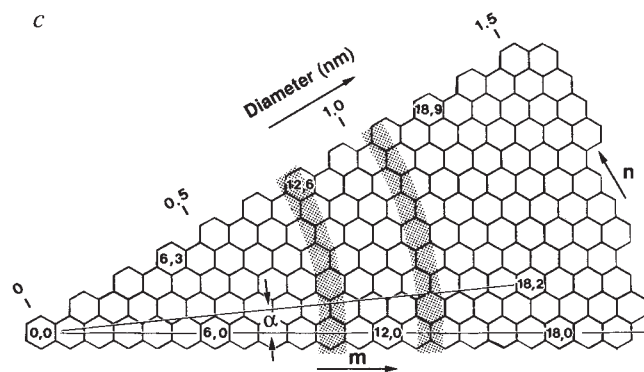
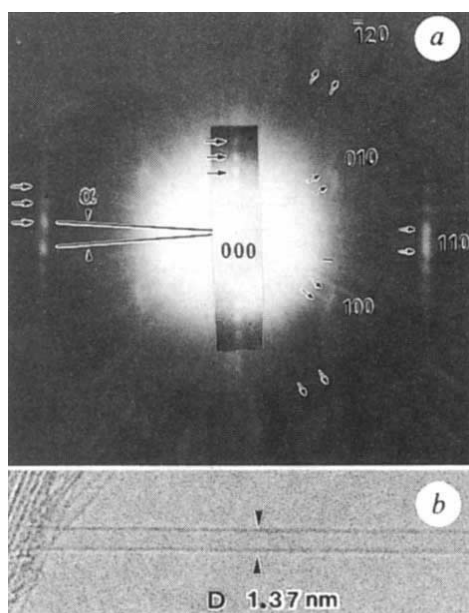


FIG. 3a, Electron diffraction pattern taken from a single-shell nanotube of diameter 1.37 nm. b, Electron micrograph of same tubule. The pattern comprises two sets of the hexagonal patterns which gives rise to splitting of the ($hk0$) spots. Each spot has periodic intensity maxima in the vertical direction, caused by Fraunhofer diffraction from the two portions of the tube imaged as two dark lines in b. c, Schematic representation of helical tubules according to Hamada's notation⁶. A tubule is represented by an index (m, n) or (m, α) , where m is m th hexagon from the origin $(0, 0)$, and n and α are defined as shown. α is a pitch angle. If the tubule diameter D and angle α are known, the tubule structure is uniquely determined. Hatched hexagons represent the tubules corresponding to the two peaks in Fig. 2.

parallel to the incident electron waves.

By knowing the tubule diameter D and its pitch angle α with respect to the fibre axis (Fig. 3a), the helicity and thus the tubule structure can be determined uniquely. To describe the tubule helicity, we follow Hamada's notation⁶ as illustrated in Fig. 3c, where a tubule can be represented by an index (m, n) . This tubule can be realized by rolling up a sheet of hexagons so as to superimpose the origin $(0, 0)$ on the hexagon (m, n) . A tubule $(m, -n)$ is chiral with the opposite handedness to (m, n) , and thus they should be optically active, but they cannot be distinguished by the diffraction experiment. The tubules which were observed frequently on the histogram (Fig. 2) have values of (m, n) in the hatched areas. On the electron diffraction pattern in Fig. 3a, α and D are measured as 7° and 1.37 nm, respectively. This tube can be indexed as $(18, 2)$ or its

enantiomer $(18, -2)$, and should behave as a semiconductor according to electronic band structure calculations^{6,16}. We should mention here that α , or helicity, varies for a given tubule diameter.

We speculate that the single-shell tubules might be the embryo for the multi-shell tubules. In our proposed model for the nanotube growth^{17,18}, the tubule ends are open so that carbon atoms are easily captured at dangling bonds, and the multi-shell tubules grow in the direction of the tube axis and also perpendicular to it. The latter growth is associated with layer-by-layer growth on the tubule surface. In the single-shell tubes, we assume that axial growth dominates over layer growth. We speculate that the iron in the present experiments acts as a homogeneous catalyst in the vapour phase. □

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1. Iijima, S. *Nature* **354**, 56–58 (1991).
2. Ajayan, P. M. & Iijima, S. *Nature* **361**, 333–334 (1993).
3. Ajayan, P. M. *et al.* *Nature* **362**, 522–523 (1993).
4. Tsang, S. C., Harris, P. J. F. & Green, M. L. *Nature* **362**, 520–522 (1993).
5. Pederson, M. R. & Broughton, J. O. *Phys. Rev. Lett.* **69**, 2687–2692 (1992).
6. Hamada, N., Sawada, S. & Oshiyama, S. *Phys. Rev. Lett.* **68**, 1579–1581 (1992).
7. Mintmire, J. W., Dunlap, B. I. & White, C. T. *Phys. Rev. Lett.* **68**, 631–634 (1992).
8. Saito, R., Fujita, F., Dresselhaus, G. & Dresselhaus, M. S. *Phys. Rev. B* **46**, 1804–1811 (1992).

9. Robertson, D. H., Brenner, D. W. & Mintmire, J. W. *Phys. Rev. B* **45**, 12592–12595 (1992).
10. Ebbesen, T. W. & Ajayan, P. M. *Nature* **368**, 220–222 (1992).
11. Ando, Y. & Iijima, S. *Jpn. J. appl. Phys.* **32**, L107–L109 (1993).
12. Oberlin, A. & Endo, M. *J. Cryst. Growth* **32**, 335–349 (1976).
13. Iijima, S. *Chem. Scripta* **14**, 117–123 (1978–79).
14. Iijima, S., Ichihashi, T. & Ando, Y. *Nature* **356**, 776–778 (1992).
15. Ajayan, P. M., Ichihashi, T. & Iijima, S. *Chem. Phys. Lett.* **202**, 384–388 (1992).
16. White, C. T., Robertson, D. H. & Mintmire, J. W. *Phys. Rev. B* **47**, 5485–5488 (1993).
17. Iijima, S., Ajayan, P. M. & Ichihashi, T. *Phys. Rev. Lett.* **69**, 3100–3103 (1992).
18. Iijima, S. *Mat. Sci. Engng B*, (in the press).

Cobalt-catalysed growth of carbon nanotubes with single-atomic-layer walls

D. S. Bethune, C. H. Kiang*, M. S. de Vries, G. Gorman, R. Savoy, J. Vazquez & R. Beyers

IBM Research Division, Almaden Research Center, 650 Harry Road, San Jose, California 95120-6099, USA

CARBON exhibits a unique ability to form a wide range of structures. In an inert atmosphere it condenses to form hollow, spheroidal fullerenes^{1–4}. Carbon deposited on the hot tip of the cathode of the arc-discharge apparatus used for bulk fullerene synthesis will form nested graphitic tubes and polyhedral particles^{5–8}. Electron irradiation of these nanotubes and polyhedra transforms them into nearly spherical carbon 'onions'⁹. We now report that covaporizing carbon and cobalt in an arc generator leads to the formation of carbon nanotubes which all have very small diameters (about 1.2 nm) and walls only a single atomic layer thick. The tubes form a web-like deposit woven through the fullerene-containing soot, giving it a rubbery texture. The uniformity and single-layer structure of these nanotubes should make it possible to test their properties against theoretical predictions^{10–13}.

The initial aim of our experiments was to produce metallofullerenes and graphite-encapsulated nanocrystals of magnetic atoms. Electrodes were prepared by boring 4-mm-diameter holes in 6-mm-diameter graphite rods and filling them with mixtures of pure powdered metals (Fe, Ni or Co) and graphite. These filled anodes (~2 at % metal) were vaporized with a current of 95–105 A in 100–500 torr of He in our arc fullerene generator. The results obtained with cobalt were unique.

When a Co-containing rod was used, what looked like spider webs formed in the chamber, draping between surfaces. The soot on the chamber walls was rubbery and could be peeled off in long strips. Normal fullerene soots (and those made with Fe- or

Ni-containing rods) are crumbly. The soot and the web material were ferromagnetic. A transmission electron microscope (TEM) image of the web material (Fig. 1) shows that the web consists of rounded soot particles a few tens of nanometres across, linked together by fine fibres. Individual threads can be traced for several micrometres. In some cases several fibres converge on a soot particle. Embedded within the soot particles are round cobalt clusters with diameters ranging from a few nanometres to roughly 20 nm. Both electron and X-ray diffrac-

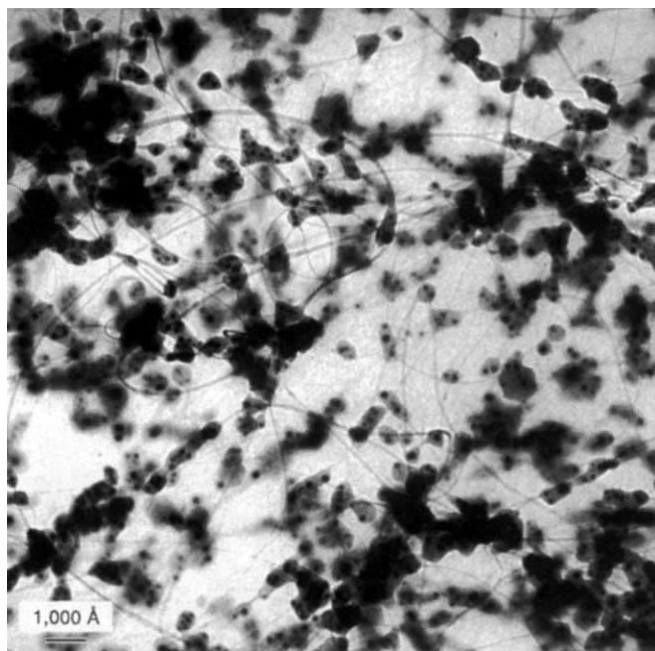


FIG. 1 Transmission electron micrograph of web-like material showing strands of thread-like fibres and cobalt clusters (dark spots) embedded in carbon soot particles. This image and those in Figs 2 and 3 were obtained using a Topcon 002B microscope operating at 200 kV and a Gatan Model 679 slow-scan camera to acquire digital images, which can then have the contrast enhanced to reveal the nanotubes.

*Affiliated with the Materials and Molecular Simulation Center, Beckman Institute, California Institute of Technology, Pasadena, California 91125, USA.

tion patterns showed that these clusters are face-centred-cubic Co. This indicates that the clusters were rapidly quenched, as Co is normally hexagonal-close-packed below 400 °C.

Scanning electron microscope (SEM) images show that the rubber soot deposits from the chamber walls contain thin fibres and soot particles similar to those in the web material, but with the particles in greater relative abundance. The carbon around the cobalt clusters consists partly of fullerenes, which can be extracted from the soot in typical amounts using toluene. Laser-desorption/laser-ionization mass-spectrometry of the raw soot showed a CoC_{60} peak, but this species was not found in a toluene extract of the soot.

A higher-magnification TEM image (Fig. 2) reveals the structures underlying the fibre and web formation. Carbon nanotubes with single-atomic-layer walls and diameters of $1.2 \pm 0.1 \text{ nm}$ are ubiquitous. The tubules apparently crossed, aggregated and tangled before being encased. Although the tubules are mostly coated with non-graphitic carbon, bare sections are also evident. Figure 3 (at still higher magnification) shows a bare nanotube with several round objects, comparable in size to fullerenes with 60–100 carbons, adhering to it. The circumference of the nanotubes would correspond to a belt of 15 or 16 edge-sharing hexagons with 0.142-nm sides.

Carbon fibres grow under diverse conditions^{14–16}. Graphitic whiskers 1–5 μm in diameter and centimetres in length can be grown on the extremely hot cathode of a carbon arc run in high-pressure argon¹⁷. Under similar conditions (but at lower pressures), tubular graphitic structures with 2–30 nm diameters and micrometre lengths form in the cathode deposits in an arc-fullerene generator^{5–7}. These nanotubes typically have walls 2–50 atomic layers thick. On the other hand, in the presence of transition-metal catalyst particles, vapour-grown carbon fibre can be produced by pyrolysing a hydrocarbon/carrier-gas mixture at temperatures between 500 °C and 1,200 °C (refs 14–16). Yacaman *et al.*¹⁸ recently reported that some fibres produced by this method resemble the hollow graphitic tubes seen in fullerene-generator cathode deposits.

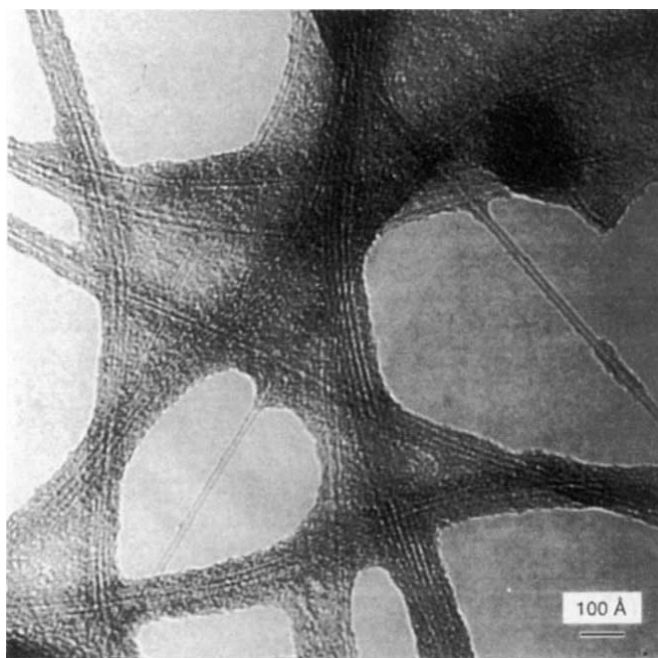


FIG. 2 TEM image at higher magnification showing details of the web-like material. Running through the deposited non-graphitic carbon are single-walled nanotubes about 1.2 nm in diameter. Bare portions of these nanotubes are also evident. The dark spot in the upper-right corner is a cobalt cluster.

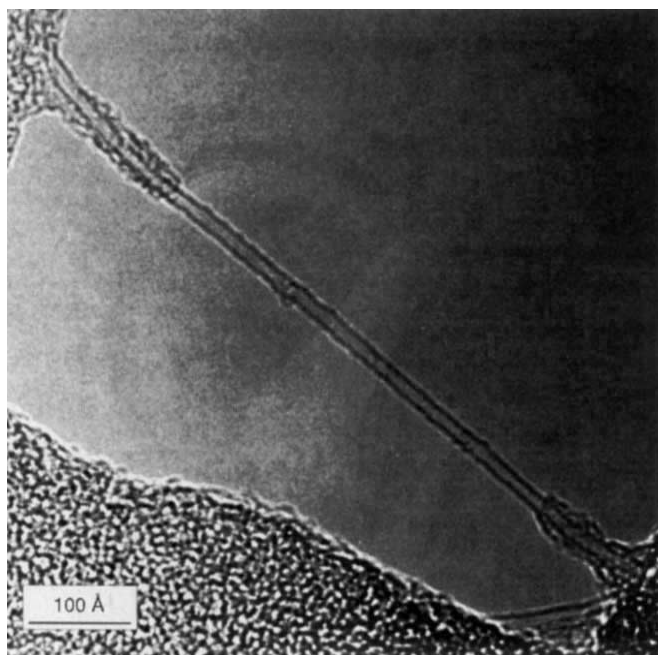


FIG. 3 TEM image of a bare section of a single-walled nanotube. The round objects adhering to the tube have diameters corresponding to fullerenes with 60–100 carbons.

In contrast to these multilayered fibres and tubes, our cobalt-catalysed nanotubes have single-atomic-layer walls and a common diameter ($\sim 1.2 \text{ nm}$). They grow from carbon vapour (with no dissociation of hydrocarbon needed) at helium pressures in the range 100–500 torr. Fullerenes form abundantly at the same time. Under the conditions we used, no fibre growth was observed using Fe, Ni or a 50:50 Ni:Cu mixture, all of which catalyse fibre growth in the presence of hot gaseous hydrocarbons^{19,20}. We believe, therefore, that cobalt plays a special role in catalysing the formation of these single-walled tubules, and suggest that a specific nucleation process may be responsible for their highly uniform diameter. For the moment the relationship between the nanotubes and the cobalt clusters is obscured by the encasing layer of carbon. The nanotubes are found in relatively cold regions of the chamber, co-condensed with (and mostly coated by) fullerene soot. It may be possible to control the amount of carbon that forms on the nanotubes by modifying the growth conditions, and the crystallinity of this carbon by post-annealing the coated nanotubes at high temperature. Such measures have been used to modify vapour-grown carbon fibres¹⁴, and could be important in attempting to exploit the uniformity of these vapour-grown nanotubes to develop new types of carbon fibres.

It may also be possible to isolate bulk quantities of bare, single-walled nanotubes. Such structures constitute a new type of all-carbon polymer. Theoretical calculations predict that they can be metallic or semiconducting, depending on their helical pitch^{10,11}. They might draw species into their interiors by capillary action, and they may be useful as catalytic containers, nanowires and solenoids²¹. The recent success of Ajayan *et al.*²² in filling nanotubes with lead supports some of these ideas. The availability of the single-walled carbon nanotubes reported here should permit characterization and further experiments. □

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1. Kroto, H. W., Heath, J. R., O'Brien, S. C., Curl, R. F. & Smalley, R. E. *Nature* **318**, 162–163 (1985).
2. Krätschmer, W., Fostiropoulos, K. & Huffman, D. R. *Chem. Phys. Lett.* **170**, 167–170 (1990).

3. Krättschmer, W., Lamb, L. D., Fostropoulos, K. & Huffman, D. R. *Nature* **347**, 354–358 (1990).
4. Meijer, G. & Bethune, D. S. *J. chem. Phys.* **93**, 7800–7802 (1990).
5. Iijima, S. *Nature* **354**, 56–58 (1991).
6. Iijima, S., Ichihashi, T. & Ando, Y. *Nature* **356**, 776–778 (1992).
7. Ebbesen, T. W. & Ajayan, P. M. *Nature* **358**, 220–222 (1992).
8. Saito, Y., Yoshikawa, T., Inagaki, M., Tomita, M. & Hayashi, T. *Chem. Phys. Lett.* **204**, 277–282 (1993).
9. Ugarte, D. *Nature* **359**, 707–708 (1992).
10. Hamada, N., Sawada, S. & Oshiyama, A. *Phys. Rev. Lett.* **68**, 1579–1581 (1992).
11. Mintmire, J. W., Duniap, B. I. & White, C. T. *Phys. Rev. Lett.* **68**, 631–634 (1992).
12. Saito, R., Fujita, M., Dresselhaus, G. & Dresselhaus, M. S. *Phys. Rev. B* **46**, 1804–1811 (1992).
13. Robertson, D. H., Brenner, D. W. & Mintmire, J. W. *Phys. Rev. B* **45**, 12592–12595 (1992).
14. Endo, M. *Chemtech* **18**, 568–576 (1998).
15. Baker, R. T. *Carbon* **27**, 315–323 (1989).
16. Tibbets, G. G. *J. Cryst. Growth* **73**, 431–438 (1985).
17. Bacon, R. J. *Appl. Phys.* **31**, 283–290 (1960).
18. Jose-Yacamán, M., Miki-Yoshida, M., Rendon, L. & Santiesteban, J. G. *Appl. Phys. Lett.* **62**, 657–659 (1993).
19. Baker, R. T. & Harris, P. S. in *Chemistry and Physics of Carbon*, Vol. 14, 83–165 (Marcel Dekker, New York, 1978).
20. Kim, M. S., Rodriguez, N. M. & Baker, R. T. *J. Catal.* **131**, 60–73 (1991).
21. Pederson, M. R. & Broughton, J. Q. *Phys. Rev. Lett.* **69**, 2689–2692 (1992).
22. Ajayan, P. M. & Iijima, S. *Nature* **361**, 333–334 (1993).

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Synchronous changes in seawater strontium isotope composition and global climate

Steven C. Clemens, John W. Farrell* & L. Peter Gromet

Geological Sciences, Brown University, Providence, Rhode Island, 02912-1846, USA

THE $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of sea water has increased gradually over the past 40 Myr, suggesting a concomitant increase in global chemical weathering rates^{1–6}. Recently, Dia *et al.*⁷ analysed a 250-kyr $^{87}\text{Sr}/^{86}\text{Sr}$ record, and found superimposed on this gradual increase higher-frequency $^{87}\text{Sr}/^{86}\text{Sr}$ variations which appeared to follow a 100-kyr cycle; this periodicity corresponds to one of the prominent cycles in the Earth's orbital parameters, which are known to modulate the patterns of solar insolation and hence climate^{8–10}. The resolution of this record was, however, insufficient to establish the phase relationship between the $^{87}\text{Sr}/^{86}\text{Sr}$ variations and global climate cycles. Here we present a high-resolution seawater $^{87}\text{Sr}/^{86}\text{Sr}$ record spanning the past 450 kyr. We find that maxima and minima in $^{87}\text{Sr}/^{86}\text{Sr}$ coincide with minima and maxima, respectively, in continental ice volume (from the SPECMAP oxygen isotope record²⁰), apparently suggesting that there was less chemical weathering in arid glacial periods than in the more humid interglacials. During glacial-interglacial transitions, however, seawater $^{87}\text{Sr}/^{86}\text{Sr}$ changes at a rate of ~ 1 p.p.m. kyr⁻¹, approximately three times that evaluated by Dia *et al.*⁷. Mass-balance calculations illustrate that simple changes in modern chemical weathering regimes cannot fully account for such rapid changes, suggesting that we need to revise current ideas about strontium reservoirs and the mechanisms for exchange between them.

To evaluate fine structure in the past 450 kyr of the seawater $^{87}\text{Sr}/^{86}\text{Sr}$ record, we measured the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios in 77 samples of marine carbonate (planktonic foraminifera) from Ocean Drilling Program/site 758 in the equatorial Indian Ocean (Fig 1, Table 1). This record can be interpreted as a global ocean signal provided that (1) seawater $^{87}\text{Sr}/^{86}\text{Sr}$ is globally homogeneous within the mixing time of the ocean, (2) biogenic calcite is precipitated in equilibrium with seawater $^{87}\text{Sr}/^{86}\text{Sr}$, and (3) the $^{87}\text{Sr}/^{86}\text{Sr}$ signal measured in planktonic foraminifera is derived from Sr in the calcite lattice and not associated phases such as

fine clays or diagenetic precipitates adhering to the skeletal calcite. Results from several studies suggest that these conditions are met. Measurements of seawater $^{87}\text{Sr}/^{86}\text{Sr}$ from Pacific and Atlantic surface and deep waters demonstrate the homogeneity of $^{87}\text{Sr}/^{86}\text{Sr}$ in the modern global ocean to within 6 p.p.m., well within the analytical error of measurement¹¹; the $^{87}\text{Sr}/^{86}\text{Sr}$ of biogenic calcite from globally distributed planktonic foraminifera and bivalves is indistinguishable from modern sea water^{3,7,11,12}; the $^{87}\text{Sr}/^{86}\text{Sr}$ of calcite from planktonic and benthic foraminifera is indistinguishable, indicating equilibrium precipitation in surface as well as deep waters¹³.

To minimize the contribution of Sr from noncarbonate material, foraminifera were prepared for $^{87}\text{Sr}/^{86}\text{Sr}$ measurement as in previous studies^{2,14,15}, by sonification in ultrapure water followed by dissolution of the calcite in weak acetic acid. To investigate further the possibility of contamination by non-carbonate phases, we cleaned a subset of our samples following more rigorous procedures^{16,17} designed to remove fine clays, oxidize organic matter and reduce precipitated metal oxides before dissolving the calcite lattice. All but one of the paired samples ($N=17$) yielded analytically indistinguishable results (Table 1).

Another approach to testing the validity of the site 758 $^{87}\text{Sr}/^{86}\text{Sr}$ record as a global signal is duplication of the results in other oceans with different sedimentary environments, using different foraminifera species. Comparison with a lower resolution record from the Pacific⁷ shows strong similarities and some differences (Fig. 1). As with the well documented ice-age cyclicity in $\delta^{18}\text{O}$ (refs 18–21), both $^{87}\text{Sr}/^{86}\text{Sr}$ records show 100-kyr fluctuations with higher values during interglacial stages and lower values during glacial stages indicating that, to first order, both sites record a global ocean signal. However, the site 758 record indicates that glacial-interglacial transitions in $^{87}\text{Sr}/^{86}\text{Sr}$ occur more or less simultaneously with changes in ice volume ($\delta^{18}\text{O}$), whereas $^{87}\text{Sr}/^{86}\text{Sr}$ in the V28-238 record appears to lead the global ice-volume signal by 10–15 kyr at deglaciations. Similarly, relative amplitude differences between the two strontium records exist within oxygen isotope stages 3 and 6. One possible explanation of these differences is that the

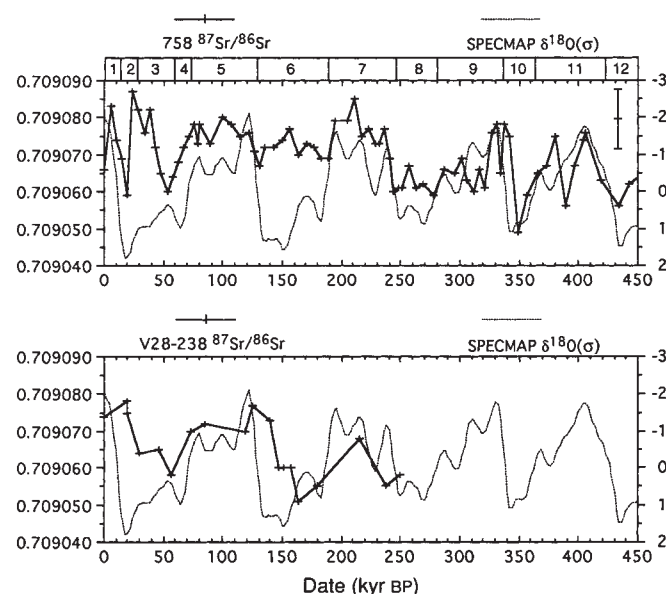


FIG. 1 Seawater $^{87}\text{Sr}/^{86}\text{Sr}$ records compared to the SPECMAP stacked $\delta^{18}\text{O}$ record²⁰ of global ice-volume. Indian Ocean site 758 (top) (error bar represents $\pm 1\sigma$) and Pacific Ocean V28-238 (bottom; ref. 7). Both records are plotted relative to an NBS-987 value of 0.710150. Oxygen isotopic stages²⁰ are labelled at the top (even numbers represent glacial intervals).

* Present address: University of British Columbia, Vancouver, British Columbia, V6T1Z4.

structure and timing of $^{87}\text{Sr}/^{86}\text{Sr}$ events were not adequately resolved in the V28-238 record. To evaluate these differences properly, records of similar resolution are required.

The high temporal resolution and length of the site 758 record enable us to examine in detail the nature of the link between seawater $^{87}\text{Sr}/^{86}\text{Sr}$ and global ice volume (Fig. 2a). Although similarities between seawater $^{87}\text{Sr}/^{86}\text{Sr}$ and $\delta^{18}\text{O}$ are striking, amplitude anomalies do exist. The $^{87}\text{Sr}/^{86}\text{Sr}$ record has comparatively high amplitude in $\delta^{18}\text{O}$ stage 3 and low amplitude in stage 6, suggesting that mechanisms not directly linked to global ice volume may also influence the $^{87}\text{Sr}/^{86}\text{Sr}$ values. Nonetheless, the 100-kyr periodicity in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ is well established by these data. We use cross-spectral analysis to quantify the link between seawater $^{87}\text{Sr}/^{86}\text{Sr}$ and $\delta^{18}\text{O}$ in terms of coherence (the degree to which amplitude variations are linearly related) and phase (the relative timing of glacial and deglacial transitions).

The spectrum of the detrended site 758 $^{87}\text{Sr}/^{86}\text{Sr}$ record shows

that variance is concentrated at the 100- and 41-kyr Earth-orbital (Milankovitch) periods known to modulate patterns of insolation and climate⁸⁻¹⁰ (Fig. 2b). Cross-spectral comparison with $\delta^{18}\text{O}$ indicates a statistically coherent (95% confidence) internal zero-phase relationship at the 100-kyr period (Fig. 2b). Examination of the main glacial-interglacial and deglacial transitions shows that seawater $^{87}\text{Sr}/^{86}\text{Sr}$ changes as rapidly as 15 p.p.m. in 15 kyr, or an average rate of ± 1 p.p.m. kyr⁻¹. Coupled with the coherent zero-phase relationship with $\delta^{18}\text{O}$, these results indicate that strontium cycling is much more dynamic than previously thought, and that there is a strong link between the glacial-interglacial cyclicity and seawater $^{87}\text{Sr}/^{86}\text{Sr}$. If the 100-kyr periodicity in $^{87}\text{Sr}/^{86}\text{Sr}$ is modelled as a simple (single exponential) linear system²², maxima in any potential forcing mechanism must occur no more than 25 kyr before maxima in seawater $^{87}\text{Sr}/^{86}\text{Sr}$ ($90^\circ/360^\circ \times 100$ kyr). Such a short interval between the forcing and the response and the rapid ± 1 p.p.m.

TABLE 1 $^{87}\text{Sr}/^{86}\text{Sr}$ results from ODP site 758

Sample ID	Depth (m)*	Age (kyr)*	$^{87}\text{Sr}/^{86}\text{Sr} \pm (1\sigma) \{ \# \} \dagger$	$^{87}\text{Sr}/^{86}\text{Sr} \pm (1\sigma) \{ \# \} \dagger$	Sample ID	Depth (m)*	Age (kyr)*	$^{87}\text{Sr}/^{86}\text{Sr} \pm (1\sigma) \{ \# \} \dagger$	$^{87}\text{Sr}/^{86}\text{Sr} \pm (1\sigma) \{ \# \} \dagger$
758A 1H-1.001	0.01	1	0.709066 (8) {2}		758A 1H-3.121	4.21	2.18	0.709075 (8) {2}	
758A 1H-1.011	0.11	6	0.709083 (8) {2}	0.709076 (8) {2}	758A 1H-3.131	4.31	223	0.709077 (8) {2}	0.709073 (8) {2}
758A 1H-1.021	0.21	10	0.709074 (8) {2}		758A 1H-3.141	4.41	228	0.709073 (8) {2}	
758A 1H-1.031	0.31	19	0.709069 (7) {3}		758A 1H-4.001	4.51	232	0.709073 (8) {2}	
758A 1H-1.041	0.41	29	0.709059 (7) {3}	0.709075 (12) {1}	758A 1H-4.011	4.61	237	0.709077 (8) {2}	
758A 1H-1.051	0.51	24	0.709087 (7) {3}	0.709085 (12) {1}	758A 1H-4.021	4.71	240	0.709069 (7) {3}	
758A 1H-1.061	0.61	29	0.709082 (7) {3}		758A 1H-4.031	4.81	244	0.709060 (8) {2}	
758A 1H-1.071	0.71	34	0.709076 (6) {4}		758A 1H-4.041	4.91	247	0.709061 (8) {2}	0.709063 (8) {2}
758A 1H-1.081	0.81	38	0.709082 (7) {3}	0.709078 (12) {1}	758A 1H-4.051	5.01	252	0.709061 (7) {3}	
758A 1H-1.091	0.91	43	0.709072 (8) {2}		758A 1H-4.061	5.11	257	0.709067 (7) {3}	
758A 1H-1.101	1.01	48	0.709065 (8) {2}		758A 1H-4.071	5.21	263	0.709061 (7) {3}	
758A 1H-1.111	1.11	53	0.709060 (8) {2}	0.709070 (8) {2}	758A 1H-4.081	5.31	268	0.709062 (8) {2}	0.709060 (8) {2}
758A 1H-1.121	1.21	58	0.709064 (8) {2}		758B 1H-3.081	5.32	269	0.709062 (8) {2}	
758A 1H-1.131	1.31	63	0.709068 (8) {2}		758B 1H-3.091	5.42	278	0.709059 (8) {2}	
758A 1H-1.141	1.41	67	0.709072 (8) {2}		758B 1H-3.101	5.52	287	0.709066 (8) {2}	
758A 1H-2.001	1.51	71	0.709075 (8) {2}		758B 1H-3.111	5.62	295	0.709065 (8) {2}	
758A 1H-2.041	1.91	76	0.709078 (8) {2}		758B 1H-3.121	5.72	301	0.709069 (8) {2}	
758A 1H-2.061	2.11	79	0.709073 (8) {2}		758B 1H-3.131	5.82	306	0.709063 (8) {2}	
758A 1H-2.071	2.21	80	0.709078 (8) {2}		758B 1H-3.141	5.92	311	0.709060 (8) {2}	
758A 1H-2.081	2.31	90	0.709073 (8) {2}		758B 1H-4.001	6.02	316	0.709066 (8) {2}	
758A 1H-2.091	2.41	99	0.709080 (8) {2}	0.709068 (12) {1}	758B 1H-4.011	6.12	321	0.709061 (8) {2}	
758A 1H-2.101	2.51	107	0.709078 (8) {2}		758B 1H-4.021	6.22	326	0.709076 (7) {3}	
758A 1H-2.111	2.61	114	0.709075 (8) {2}	0.709075 (8) {2}	758B 1H-4.031	6.32	331	0.709078 (8) {2}	
758A 1H-2.121	2.71	122	0.709076 (8) {2}		758B 1H-4.041	6.42	334	0.709065 (8) {2}	
758A 1H-2.131	2.81	126	0.709071 (8) {2}		758B 1H-4.051	6.52	338	0.709078 (8) {2}	
758A 1H-2.141	2.91	131	0.709067 (8) {2}		758B 1H-4.061	6.62	341	0.709075 (8) {2}	0.709074 (12) {1}
758A 1H-3.001	3.01	135	0.709072 (8) {2}		758B 1H-4.071	6.72	349	0.709049 (8) {2}	0.709051 (12) {1}
758A 1H-3.011	3.11	142	0.709072 (7) {3}		758B 1H-4.081	6.82	357	0.709059 (8) {2}	
758A 1H-3.021	3.21	149	0.709074 (7) {3}		758B 1H-4.091	6.92	365	0.709065 (8) {2}	
758A 1H-3.031	3.31	157	0.709077 (7) {3}	0.709066 (12) {1}	758B 1H-4.101	7.02	373	0.709067 (8) {2}	
758A 1H-3.041	3.41	164	0.709070 (7) {3}		758B 1H-4.111	7.12	381	0.709075 (8) {2}	0.709062 (8) {2}
758A 1H-3.051	3.51	171	0.709073 (8) {2}		758B 1H-4.121	7.22	388	0.709056 (8) {2}	
758A 1H-3.061	3.61	177	0.709072 (8) {2}		758B 1H-4.131	7.32	396	0.709067 (8) {2}	
758A 1H-3.071	3.71	183	0.709069 (8) {2}	0.709058 (8) {2}	758B 1H-4.141	7.42	404	0.709074 (8) {2}	
758A 1H-3.081	3.81	189	0.709069 (12) {1}		758A 2H-1.001	7.43	405	0.709076 (12) {1}	
758A 1H-3.091	3.91	194	0.709079 (8) {2}		758A 2H-1.011	7.53	420	0.709063 (8) {2}	
758A 1H-3.101	4.01	205	0.709079 (8) {2}		758A 2H-1.021	7.63	434	0.709056 (8) {2}	0.709073 (8) {2}
758A 1H-3.111	4.11	211	0.709085 (8) {2}	0.709082 (8) {2}	758A 2H-1.031	7.73	443	0.709062 (8) {2}	
					758A 2H-1.041	7.83	452	0.709064 (8) {2}	
Global Sr budget		Age (kyr)	$^{87}\text{Sr}/^{86}\text{Sr} \dagger$	moles Sr yr ⁻¹				Reference	
Sea water		modern	0.709078	(1.25x10 ¹⁷ moles)				11	
Global rivers		modern	0.7119	3.33(±1)x10 ¹⁰				29	
Hydrothermal		modern	0.7035	1.51(±0.38)x10 ¹⁰				29	
Deep-sea carbonate		modern	0.7084	0.34x10 ¹⁰				29	
Subaerially exposed reefs		glacial	0.709073	8.00x10 ¹⁰				this work 24, 32	

* Depths and ages from ref. 33. Ages are based on correlation of $\delta^{18}\text{O}$ (*Globogerioides sacculifer*) from site (5° 23' N, 90° 21' E; 2,925 m water depth) to the SPECMAP stacked $\delta^{18}\text{O}$ record²⁰.

† Strontium isotopes were measured on the acetic acid-soluble portions of five hand-picked and ultrasonically cleaned tests of the planktonic foraminifera *Globobulimina menardii* (>425 µm). Samples were analysed for $^{87}\text{Sr}/^{86}\text{Sr}$ by thermal ionization mass spectrometry with a Finnigan-MAT 261 mass spectrometer using static multiple collection with simultaneous collection of mass 85 to monitor for the presence of Rb. The filament current was raised over a period of 45 min to yield a stable ^{86}Sr signal of $\sim 4 \times 10^{11}$ A. We did not detect a statistically resolvable signal at mass 85, and any possible corrections are of the order of $2-3 \times 10^{-6}$. Results were normalized for mass fractionation to a $^{86}\text{Sr}/^{88}\text{Sr}$ ratio of 0.1194 according to an exponential fractionation law. The site 758 $^{87}\text{Sr}/^{86}\text{Sr}$ ratio measurements were made between May and October 1992 during which time one or more aliquots of the standard NBS-987 were analysed with each spectrometer run (6–12 samples). The external error associated with NBS-987 replicate analyses is ± 0.000012 (1σ, N=112). We report results relative to an NBS-987 value of 0.710150. A typical analysis consisted of 14 blocks of 15 scans lasting 32 seconds each (210 ratio counts). To estimate sample precision, which accounts for sample inhomogeneity and sample processing, we made 91 replicate analyses from raw sample (168 total analyses). Each replicate was passed through the entire chemical procedure. The resulting standard deviation³⁴ (± 0.000011 ; 1σ) agrees well with the value calculated from standards. The $\pm 1\sigma$ reported with each sample is calculated as the long-term external error (± 0.000012) divided by $n^{0.5}$ where n is the number {#} of replicate analyses from raw sample (typically 2). Two full procedural blanks run during data collection contained 32 and 96 pg of Sr.

‡ Same as in † but using 10 foraminifera and the cleaning method of Boyle^{16,17}. These data are not included in the larger data set (†).

§ Seawater ratio adjusted to an NBS-987 value of 0.710150. River, deep-sea carbonates, and hydrothermal values are relative to an E&A standard value of 0.7080 (given a precision of ± 0.0001 , correction to an NBS-987 value of 0.710150 would be artificial). The reef ratio is the average of all site 758 interglacial-age data points.

kyr⁻¹ rate at transitions are surprising given the current estimate of 2.5 Myr for the residence time of strontium in the oceans³.

Simple mass-balance calculations illustrate that extreme changes in the flux and/or isotopic composition of modern strontium sources are required to change seawater ⁸⁷Sr/⁸⁶Sr by 1 p.p.m. kyr⁻¹. Modern seawater ⁸⁷Sr/⁸⁶Sr is determined by riverine input from the continents (chemical weathering products), in addition to inputs from the upper mantle (exchange at ocean-ridge hydrothermal vents) and deep-sea carbonates (diagenesis and dissolution) (Table 1). On glacial-interglacial timescales hydrothermal inputs have been assumed constant and the contribution from deep-sea carbonate dissolution is too

small to change seawater ⁸⁷Sr/⁸⁶Sr significantly^{3,7}. But the ~120-m drop in global sea level associated with the growth of continental ice-sheets during glacial periods²³ results in the subaerial exposure of large shallow-water platform carbonates (reefs). We estimate that meteoric diagenesis of these carbonates²⁴ may contribute as much as 8×10^{10} mol Sr yr⁻¹ to the glacial ocean, about twice the modern river contribution (Table 1). Despite the large flux, this factor only buffers rapid change in seawater ⁸⁷Sr/⁸⁶Sr because the average isotopic composition of reefal carbonates is that of sea water during the immediately preceding interglacial cycles when they formed. These factors suggest that changes in global chemical weathering rates are probably responsible for controlling cyclicity in seawater ⁸⁷Sr/⁸⁶Sr on glacial-interglacial timescales^{3,7}.

The extent to which the flux and/or isotopic composition of continental weathering products (river input) must differ from modern values in order to accommodate the observed decrease of 1 p.p.m. kyr⁻¹ from interglacial (such as modern) to glacial times can be calculated from the general equation²⁵⁻²⁷

$$N \, dR_{sw}/dt = J_r (R_r - R_{sw}) + J_h (R_h - R_{sw}) + J_c (R_c - R_{sw})$$

where N is the number of moles of strontium in the ocean, and $R_{sw,r,h,c}$ and $J_{r,h,c}$ are the ⁸⁷Sr/⁸⁶Sr and flux (mol Sr yr⁻¹) of seawater, river, hydrothermal and carbonate input (deep-sea or reef), respectively (Table 1). Solving independently for J_r and R_r and using the observed dR_{sw}/dt of -1 p.p.m. kyr⁻¹, we calculate that either riverine Sr flux must decrease to zero, or that average riverine ⁸⁷Sr/⁸⁶Sr must decrease by 0.004 (to 0.7079), or some combination thereof, to drive modern seawater ⁸⁷Sr/⁸⁶Sr to glacial-age values. Uncertainties in the flux terms (Table 1) have minimal impact on the magnitude of these results.

We consider the cessation of strontium flux from the continents during glacial intervals to be physically inconceivable. Current estimates from atmospheric general circulation models indicate only a ~10% decrease in glacial-age precipitation²⁸ and, presumably, a similarly small decrease the strontium flux term. An average glacial-age riverine ⁸⁷Sr/⁸⁶Sr of ~0.7079 is also implausible. Such low values are currently found only in rivers draining young arc, continental rift and Cretaceous limestone settings, which represent only 3% of the global strontium input to the modern ocean²⁹. Thus changes in chemical weathering, as understood in the modern (interglacial) context, seem to offer little possibility of explaining the full extent of the rapid glacial-deglacial shifts observed in seawater ⁸⁷Sr/⁸⁶Sr.

This difficulty suggests an inadequate understanding of the various strontium reservoirs and the mechanisms for exchange among them, at least on glacial-interglacial timescales. Other factors will have to be explored in future work, including the existence of glacial-age strontium sources or sinks not yet accounted for such as groundwater runoff³⁰ from Jurassic and Cretaceous-age carbonate reservoirs (⁸⁷Sr/⁸⁶Sr ≈ 0.7075; see for example ref. 31) during sea-level lowstands; glacial-interglacial changes in chemical weathering processes for which no modern analogues are known; and a possible breakdown of one or more of the underlying assumptions, such as the homogeneity of the the global ocean with respect to ⁸⁷Sr/⁸⁶Sr during highly dynamic glacial-deglacial transitions, or the invariant nature of hydrothermal flux over these timescales. In any case, the site 758 record demonstrates the highly dynamic nature of the strontium cycle and indicates a need for further research, particularly over glacial-interglacial timescales. □

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1. Raymo, M. E., Ruddiman, W. F. & Froelich, P. N. *Geology* **16**, 649–653 (1988).
2. Capo, R. C. & DePaolo, J. D. *Science* **249**, 51–55 (1990).
3. Hodell, D. A., Mead, G. A. & Mueller, P. A. *Chem. Geol.* **80**, 291–307 (1990).
4. Raymo, M. E. & Ruddiman, W. F. *Nature* **359**, 117–122 (1992).
5. Caldeira, K. *Nature* **357**, 578–581 (1992).
6. Palmer, M. R. & Edmond, J. M. *Geochim. cosmochim. Acta* **56**, 2099–2111 (1992).
7. Dia, A. N., Cohen, A. S., O'Nions, R. K. & Shackleton, N. J. *Nature* **356**, 786–788 (1992).

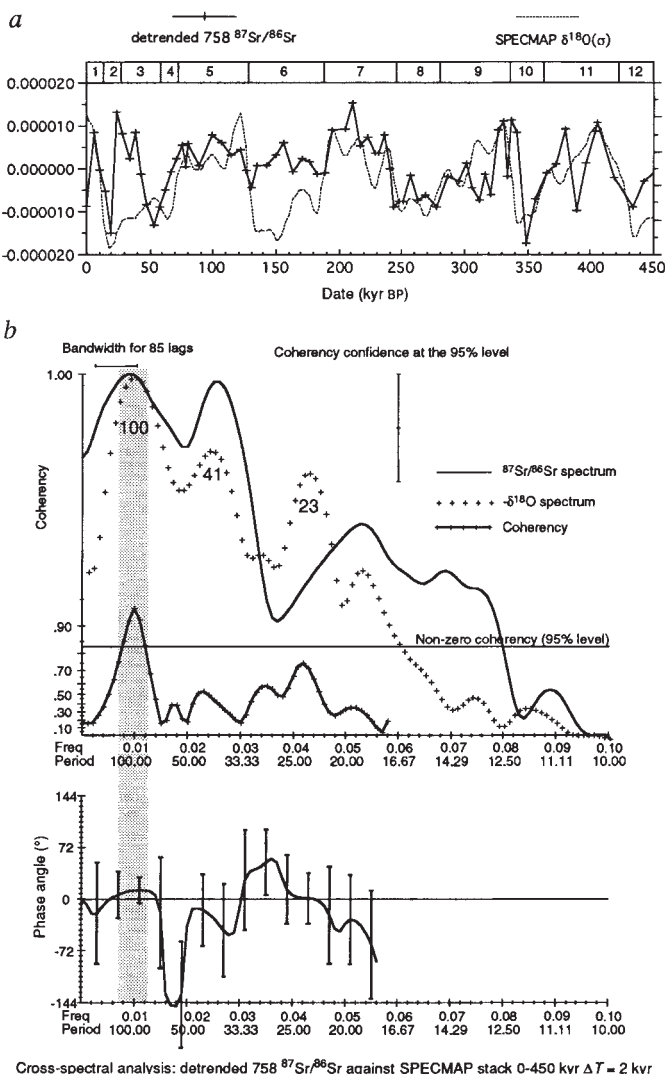


FIG. 2 Comparison between the detrended site 758 ⁸⁷Sr/⁸⁶Sr and SPECMAP stacked ^δ18O records. *a*, Same as in Fig 1 (top) except the long-term Cenozoic increase has been removed by subtraction of a simple linear fit to the ⁸⁷Sr/⁸⁶Sr data to aid comparison with the global ice-volume record. *b*, Spectral comparison. Spectral densities (σ^2/f , where frequency, f , = 1/period) are normalized and plotted on log scales (not shown). As with the ^δ18O record, the ⁸⁷Sr/⁸⁶Sr record has concentrations of variance at the 100- and 41-kyr Earth-orbital (Milankovitch) periods. The coherence spectrum (y -axis; solid line with plus signs) is plotted on a hyperbolic arctangent scale. The solid horizontal line indicates confidence at the 95% level. Phase angles (α , in degrees) are translated into years at any period (ϕ) as follows: $(\alpha/360^\circ \times \phi)$. Statistically significant coherence and a zero-phase relationship at the 100-kyr period indicate a linear relationship between increased global ice-volume and decreased seawater ⁸⁷Sr/⁸⁶Sr.

8. Berger, A. L. *Quat. Res.* **9**, 139–167 (1978).
9. Berger, A. *Quat. Int.* **2**, 1–14 (1989).
10. Imbrie, J. *et al.* *Paleoceanography* **7**, 701–738 (1992).
11. Capo, R. C. & DePaolo, D. J. *Eos* **73**, 272 (1992).
12. Elderfield, H. *Palaeogeog. Palaeoclimatol. Palaeoecol.* **57**, 71–90 (1986).
13. Beets, C. J. *Proc. ODP Sci. Res.* **117**, 459–463 (1991).
14. DePaolo, D. J. & Imgram, B. L. *Science* **227**, 938–941 (1985).
15. McKenzie, J. A., Hodell, D. A., Mueller, P. A. & Mueller, D. W. *Geology* **16**, 1022–1025 (1988).
16. Boyle, E. A. *Earth planet. Sci. Lett.* **53**, 11–35 (1981).
17. Boyle, E. A. & Keigwin, L. D. *Earth planet. Sci. Lett.* **76**, 135–150 (1986).
18. Hays, J. D., Imbrie, J. & Shackleton, N. J. *Science* **194**, 1121–1132 (1976).
19. Imbrie, J. & Imbrie, J. Z. *Science* **207**, 943–953 (1980).
20. Imbrie, J. *et al.* in *Milankovitch and Climate, Part 1* (eds Berger, A. L., Imbrie, J., Hays, J., Kukla, G. & Saltzman, B.) 269–305 (Reidel, Hingham, 1984).
21. Imbrie, J. *J. geol. Soc. London* **142**, 417–432 (1985).
22. Jenkins, G. M. & Watts, D. G. *Spectral Analysis and its Applications* (Holden Day, Oakland, 1968).
23. Fairbanks, R. G. *Nature* **342**, 637–642 (1989).
24. Schlanger, S. O. *Strontium Storage and Release during Deposition and Diagenesis of Marine Carbonates Related to Sea-Level Variations* 329–339 (Kluwer, Boston, 1988).
25. Brass, G. W. *Geochim. cosmochim. Acta* **40**, 721–730 (1976).
26. Hodell, D. A., Mueller, P. A., Mackenzie, J. A. & Mead, G. A. *Earth planet. Sci. Lett.* **92**, 165–178 (1989).
27. Richter, F. M., Rowley, D. B. & DePaolo, D. J. *Earth planet. Sci. Lett.* **109**, 11–23 (1992).
28. Kutzbach, J. E. & Guetter, P. J. *J. atmos. Sci.* **43**, 1726–1759 (1986).
29. Palmer, M. R. & Edmond, J. M. *Earth planet. Sci. Lett.* **92**, 11–26 (1989).
30. Chaudhuri, S. & Clauer, N. *Chem. Geol.* **59**, 293–303 (1986).
31. Starinsky, A., Bielecki, M. & Lazar, B. *Earth planet. Sci. Lett.* **47**, 75–80 (1980).
32. Opdyke, B. N. & Walker, J. C. G. *Geology* **20**, 733–736 (1992).
33. Farrell, J. W. & Janacek, T. R. in *Proc. ODP Sci. Results*, 121 (eds Peirce, J. *et al.*) (Ocean Drilling Program, College Station, Texas, 1991).
34. Popp, B. N., Podosek, F. A., Brannon, J. C., Anderson, T. F. & Pier, J. *Geochim. cosmochim. Acta* **50**, 1321–1328 (1986).

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Tephra from the Minoan eruption of Santorini in sediments of the Black Sea

F. Gulchard††, S. Carey†, M. A. Arthur†‡, H. Sigurdsson† & M. Arnold*

* Centre des Faibles Radioactivités (CFR), Laboratoire mixte CEA-CNRS, Domaine du CNRS, BP 1, F-91198 Gif-sur-Yvette Cedex, France

† Graduate School of Oceanography (GSO), University of Rhode Island, Narragansett, Rhode Island 02882-1197, USA

‡ Department of Geosciences, Pennsylvania State University, University Park, Pennsylvania 16802, USA

THE explosive eruption of Santorini volcano in the Aegean Sea about 3,300 years ago is of considerable archaeological and volcanological significance^{1–5}. Here we report the discovery of tephra from this Minoan event in laminated sediments of the Black Sea. This finding provides constraints on the distribution of debris from the eruption. We estimate a minimum fallout area of 2×10^6 km² extending from the Black Sea in the north to the southeastern Mediterranean Sea. The main dispersal axis trends through southern Turkey, in agreement with other studies of Minoan tephra^{6,7}. The tephra deposits should provide a useful reference horizon for assessing the chronology of Black Sea sediments, which has been much debated^{8–15}.

A thin ash layer has been detected in four sediment cores from the southern Black Sea (Fig. 1). It is undisturbed, pale in colour, and mostly made up of angular glass shards (~150 µm in longest dimension), with minor amounts of plagioclase and pyroxene crystals (<14%). Glass shards in cores GGC-79, GGC-59 and GGC-24 all have a refractive index of 1.510. The stratigraphy of the cores is similar to that described previously in this area^{8,9} and consists of the following units from the top: unit I, Holocene coccolith-bearing laminated marl; unit II, a sapropelic finely laminated mud; and unit III, alternating dark- and light-coloured lutite.

The tephra layer occurs within the upper third of unit II in all four cores. As unit II is a chronostratigraphic sediment sequence

throughout the Black Sea^{9,12,15,18}, the ash layers in the four cores were deposited at roughly the same time. The ash is not washed in from Turkey, as the constant thickness indicates that the amount deposited does not depend on distance from the coast (Table 1). Moreover, no glass shards are found in the sediments directly above or below the ash horizon, indicating a short time interval for the sedimentation and a lack of benthic biological activity.

Glass shards from core GGC-79 were analysed for major elements using an electron microprobe. Glass shards are chemically homogeneous, as shown by the low analytical standard deviations, and rhyodacitic in composition (Table 2). The K₂O content and the K₂O/Na₂O ratio (0.7) of the glass indicate that it was derived from a calc-alkaline volcanic centre. The only areas that could have produced magmas of this chemical affinity in the past are nearby volcanic centres in Turkey¹⁹, the Hellenic arc of the southern Aegean Sea and Aeolian islands of the southern Tyrrhenian Sea^{20–22}.

The age of the tephra layer can be constrained by accelerator mass spectrometry (AMS) carbon dating and lamina counting from Black Sea sediments. An AMS radiocarbon age of 3,200 years before present (BP) was obtained for the unit I/II boundary¹², whereas lamina counting suggests 1,633 yr BP with an uncertainty of less than 7% (E. Neff, manuscript in preparation). The unit II/III boundary has yielded a maximum uncorrected ¹⁴C age of 8,660 ± 150 yr BP from a core in the central Black Sea²³. The ash layer deposition thus occurred between 1,633 and 8,660 yr BP.

The largest ash-producing eruption of calc-alkaline composition within the constrained age range is the Minoan eruption of Santorini. This event discharged ~30 km³ of rhyodacite magma^{5–7}. The specific age of this event is still under debate^{2,3}. Radiocarbon data, however, strongly support a date in the seventeenth century BC, and a safe guess for the age of the eruption would be 1,650 ± 50 yr BC (between 3,290 and 3,410 yr

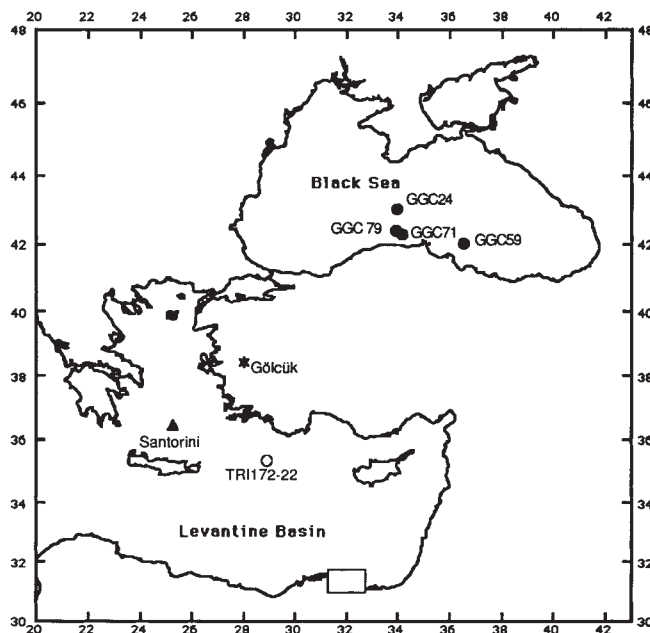


FIG. 1 Map of the eastern Mediterranean and the Black Sea. Locations of Santorini island (▲), core locations containing the Minoan ash layer in the Black Sea (●), the Levantine basin core TR172-22 chosen as a reference for ash chemistry (○), and the area of the Nile delta where glass shards have been correlated to the Minoan ash²⁹ (open rectangle) are indicated. One distal Minoan land deposit in Turkey³³ is also shown (☆).

TABLE 1 Cores with the Minoan ash

Core	Latitude N	Longitude E	Water depth (m)	Distance from Santorini (km)	Ash layer depth in cores (cm)	Ash thickness (μm)	Largest size (μm)
GGC79	42° 19.39	34° 00.94	707	1,043	74.2	150	105
GGC71	42° 12.02	34° 06.33	411	1,041	92.2	188	106
GGC24	42° 59.00	34° 00.37	2,195	1,096	47.7	122	105
GGC59	41° 58.54	36° 33.30	600	1,201	77	n.m.	105
TR172-22	35° 19.30	29° 01.20	3,150	362	13.5–18	55,000	210
Nile delta	31° 30.00	32° 00.00	n.i.	800	Dispersed	Dispersed	n.i.

For the Black Sea: GGC cores were collected during a joint US Turkish expedition³⁴. Levantine core TR172-22 is from the core collection of the University of Rhode Island. For Nile delta cores, area is deduced from the map shown in ref. 29. Largest size refers to glass shards, n.m. or n.i.: not measured or no indication.

BP). Here we have adopted an age of $3,355 \pm 32$ yr BP based on the average of four samples from archaeological excavations at Akrotiri on Santorini island¹⁶.

The composition of glass shards in the Black Sea layer is strikingly similar to those of Minoan land-based pyroclastic deposits on Santorini²⁴ and of Minoan tephra in various deep-sea cores of the eastern Mediterranean²⁴, including core TR172-22 (Table 2). In particular, the normalized proportions of K_2O , FeO and $\text{CaO}+\text{MgO}$ overlap completely for the land-based, Mediterranean and new Black Sea samples (Fig. 2). Some analyses of samples from Turkish volcanic centres^{19,25–27} overlap compositionally with the Minoan and Black Sea tephra (Fig. 2), but recent K/Ar measurements on late basaltic flows from former calderas in Central Turkey give ages greater than 60 kyr BP, and no subsequent ash products of rhyodacitic composition have been found (P.-Y. Gillot, personal communication). An Italian source can also be rejected because no important deposit of this composition occurred in the time interval concerned^{20,22}.

The distribution of Minoan tephra in deep-sea sediments of the eastern Mediterranean has previously been used to infer a southeasterly dispersal axis of fallout for this event⁴. But a more recent reconstruction places the main dispersal axis in a more easterly direction, trending through southern Turkey^{6,7}. Our correlation of the Black Sea tephra layer with the Minoan eruption is thus consistent with the age limits, the major element composition of glass shards, and new data on the more easterly dispersal pattern.

The presence of the Minoan layer in the Black Sea shows the great size of the dispersal area of ash from the volcano. The volume of magma erupted suggests that the event occurred over 3 to 4 days⁵. Much of the tephra fallout probably originated as co-ignimbrite ash⁴. Source plumes for this type of fallout are thought to be considerably larger than plinian eruption columns, which are fed from a point source²⁸. With the discovery of Minoan tephra in sediments from the Nile delta²⁹ and in the Black Sea, it is now apparent that ash fallout encompassed an area of great width (1,300 km) relative to the distance downwind. An estimate of $2.2 \times 10^6 \text{ km}^2$ can be made for the area affected by ash fallout. Nevertheless some published compositional data in the Nile delta²⁹ are not consistent with the Minoan occurrence (Fig. 2): excluding this region, a conservative estimate of $2 \times 10^6 \text{ km}^2$ is obtained.

The interbedded Minoan horizon can be used to help refine the chronology of the recent sediments in the Black Sea. AMS ^{14}C ages of the total combustible organic carbon from the ash layer are $4,450 \pm 120$ yr BP for core GGC-79 and $4,830 \pm 120$ yr BP for GGC-71 ($\pm 2\sigma$). The data indicate that the organic matter is $1,095 \pm 124$ yr (GGC-79) and $1,475 \pm 124$ yr (GGC-71) older than the Minoan event. These ^{14}C ages reflect the combined ^{14}C age of marine organic carbon and 'old' terrestrial organic carbon. To resolve the terrestrial input, we measured the concentration and carbon isotope ratio of organic carbon very close to the Minoan

horizon in GGC-79 (at 74.8 cm) and in GGC-24 (at 48 cm), because no more material from GC-71 was available. The organic carbon contents in these cores are 3.50% and 8.15% respectively ($2\sigma < 5\%$), and $\delta^{13}\text{C}$ organic ratios are -25.29‰ and -25.11‰ respectively ($\delta^{13}\text{C} = (^{13}\text{C}/^{12}\text{C})_{\text{sample}} / (^{13}\text{C}/^{12}\text{C})_{\text{standard}} - 1$, where standard is the PDB; $2\sigma < 0.10\text{‰}$). This value of $\sim -25\text{‰}$ could reflect either equal contributions of pure marine and pure terrestrial sources (estimates of end-member values are -23‰ and -27‰ , respectively¹⁸) or a fluctuation in carbon isotopic fractionation by Black Sea phytoplankton.

Nevertheless we believe it to be unlikely that terrestrial organic carbon is present at the Minoan horizon, because this fraction is probably trapped near the continent^{18,30}: no organic $\delta^{13}\text{C}$ east–west gradient was detected in the Black Sea sediment and organic $\delta^{13}\text{C}$ profiles have thus been proposed as a stratigraphic tool in this basin¹⁸. Similarly no north–south $\delta^{13}\text{C}$ gradient was apparent in the $\delta^{13}\text{C}$ data presented here (continental slope for GGC-79 and deep basin for GGC-24). These data support the hypothesis that the carbon at the ash layer depth at these core sites is purely of marine origin.

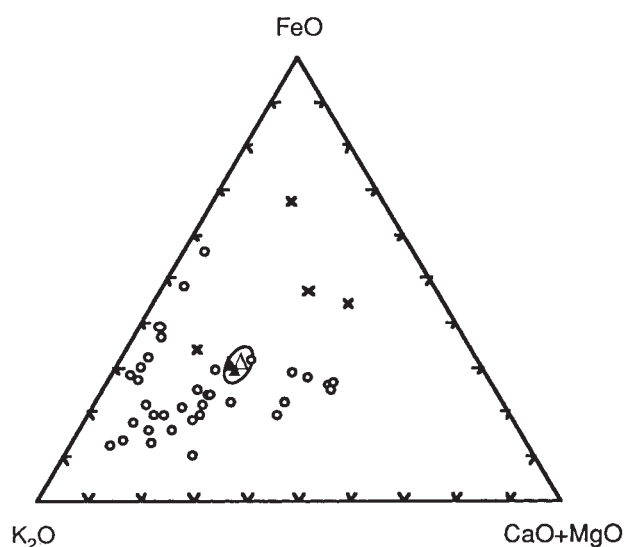


FIG. 2 Ternary plot of FeO – K_2O – $(\text{CaO}+\text{MgO})$. $\blacktriangle$: Marine tephra layers of Minoan age from the eastern Mediterranean²⁴, Minoan ash layer from Levantine core TR172-22 (this work) and ash layer in Black Sea core GGC-79 (this work). $\triangle$: For land deposit: data from Santorini island (upper pumice serie)²⁴ and Gölcük deposit from Turkey³³. The overlap of the triangles is so close that only three of the points are visible. The small oval corresponds to the field of land deposit and marine samples of Minoan age as shown in ref. 20. $\times$: Glass shards from Nile delta cores²⁹. $\circ$: Selected data from lavas of Turkey with $\text{SiO}_2 > 67\%$ ^{19,25–27}.

TABLE 2 Composition of glass shards from Levantine and Black Sea cores

Core	Number of shards	Na ₂ O	MgO	Al ₂ O ₃	SiO ₂	P	S	K ₂ O	CaO	TiO ₂	MnO	FeO*	Cl
TRI172-22	10	4.99 (0.46)	0.12 (0.13)	13.61 (0.21)	73.02 (0.47)	0 (0.01)	0.17 (0.12)	3.37 (0.16)	1.4 (0.15)	0.3 (0.14)	0.11 (0.13)	2.13 (0.25)	0.78 (0.13)
GGC-79	16	5.01 (0.28)	0.23 (0.12)	13.69 (0.28)	72.93 (0.56)	0.01 (0.02)	0.19 (0.13)	3.33 (0.11)	1.33 (0.1)	0.32 (0.1)	0.07 (0.07)	2.08 (0.22)	0.8 (0.1)
Minoan	141	4.9	0.26 (0.02)	13.7 (0.4)	72.5 (1.1)	n.i.	n.i.	3.1 (0.2)	1.3 (0.1)	0.26 (0.1)	n.i.	1.9 (0.1)	n.i.
Minoan (Bo)	6		0.24 (0.02)	13.6 (0.06)	70.6 (0.18)	n.i.	n.i.	3.12 (0.04)	1.35 (0.01)	0.26 (0.02)	n.i.	1.74 (0.06)	n.i.

Shards from Minoan tephra in Levantine core TRI172-22 and in Black Sea core GGC-79. For GGC-79 the ash layer was carefully sampled, an oily fraction was discarded by methanol, the sample was washed with water, sieved at 25 µm and dried, then individual glass shards were picked for electron microprobe analysis. Major element analyses were made on individual glass shards with a scanning electron microscope, coupled with an energy-dispersive X-ray spectrometer. Standard deviation (2σ) in parenthesis. FeO: total iron expressed as FeO. Minoan data refers to marine tephra layers of Minoan age from eastern Mediterranean area²⁴. Minoan (Bo) refers to land deposit on Santorini island ("upper pumice serie")²⁴. n.i.: no information.

Therefore, we propose to use the mean ¹⁴C age from the ash layer of the two cores, and to consider age difference of 1,285±460 yr between this and the Minoan event as representative of the inferred ¹⁴C age of Black Sea surface waters at the time of deposition.

It is inappropriate to assume the same Δ¹⁴C correction for a mid-latitude open ocean site (~400 yr)¹⁷ and a nearly closed basin such as the Black Sea. Moreover, the calibration of 1,285±460 yr which we infer for Black Sea surface water at 3,355 yr BP is in good agreement with the pre-bomb age of about 1,430 yr for surface water estimated from a three-box model³¹. The Black Sea circulation was presumably close to steady state well before the Minoan outburst at 3,355 yr BP. The start of the Mediterranean input occurred between 7,000 and 12,000 yr BP^{8,9,15}; it then took 2,000 yr or less before all the previous deep water was exchanged³². Therefore, the inferred Δ¹⁴C correction of ~1,280 yr is applicable over the past several thousand years since the renewal of deep water was completed.

This study underlines the delicate use of ¹⁴C data in the Black Sea stratigraphy. The Minoan horizon, extending eastwards over a large part of Asia Minor from its source, provides an important calibration horizon for the chronology of recent Black Sea sediments. □

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1. Marinatos, S. *Antiquity* **13**, 425–439 (1939).
2. Manning, S. W. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 29–41 (Thera Foundation, London, 1990).
3. Kuniholm, P. I. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 13–18 (Thera Foundation, London, 1990).
4. Watkins, N. D., Sparks, R. S. J., Sigurdsson, H., Huang, T. C. & Federman, A. *Nature* **271**, 122–126 (1978).
5. Sparks, R. S. J. & Wilson, C. J. N. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 89–99 (Thera Foundation, London, 1990).
6. Sigurdsson, H., Carey, S. & Devine, J. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 100–112 (Thera Foundation, London, 1990).
7. Pyle, D. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 113–121 (Thera Foundation, London, 1990).
8. Ross, D. A., Degens, E. T. & MacIvaine, J. *Science* **170**, 163–165 (1970).
9. Ross, D. A. & Degens, E. T. in *The Black Sea: Geology, Chemistry and Biology* (eds Ross, D. A. & Degens, E. T.) 183–199 (Am. Assoc. Pet. Geol. Mem., 1974).
10. Degens, E. T. *et al. Neues Jb. Geol. paläontol. Monat.* **5**, 65–86 (1980).
11. Calvert, S. E., Karlén, R. E., Toolin, L. J., Donahue, D. J. & Southon, J. R. *Nature* **350**, 692–695 (1991).
12. Jones, G. A. *Radiocarbon* **33**, 211–213 (1991).
13. Crusius, J. & Anderson, R. F. *Paleoceanography* **7**, 215–227 (1992).
14. Crusius, J. & Anderson, R. F. *Geochim. cosmochim. Acta* **55**, 327–333 (1991).
15. Hay, B. J., Arthus, M. A., Dean, W. E., Neff, E. D. & Honjo, S. *Deep-Sea Res.* **38**, Suppl. 2A, S1211–S1235 (1991).
16. Friedrich, W., Wagner, P. & Tauber, H. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 188–196 (Thera Foundation, London, 1990).
17. Bard, E. *Paleoceanography* **3**, 635–645 (1988).
18. Calvert, S. E. & Fontugne, M. R. *Chem. Geol. (Isotope Geosci. Section)* **66**, 315–322 (1987).
19. Innocenti, F., Mazzuoli, R., Pasquare, G., Radicati di Brozolo, F. & Villari, L. *Geol. Mag.* **112**, 349–360 (1975).
20. Keller, J. in *Tephra Studies* (eds Self, R. S. J. S. & Sparks, S.) 227–244 (Reidel, Dordrecht, 1981).

21. Keller, J., Rehren, T. H. & Stadlbauer, E. in *Thera and the Aegean World III* (ed. Hardy, D. H.) 13–26 (Thera Foundation, London, 1990).
22. Paternite, M., Guichard, F., & Labeyrie, J. J. *Volcan. geotherm. Res.* **34**, 153–172 (1988).
23. Calvert, S. E., Vogel, J. S. & Southon, J. R. *Geology* **15**, 918–921 (1987).
24. Federman, A. N. & Carey, S. *Quat. Res.* **13**, 160–171 (1980).
25. Innocenti, F., Mazzuoli, R., Pasquare, G., Serri, G. & Villari, L. *Geol. Rundsch.* **69**, 292–322 (1980).
26. Aydar, E. *Les Laves du Quaternaire de Cappadoce (Turquie): Volcanologie et Petrologie* (Mémoire DEA, Clermont-Ferrand Univ. 1989).
27. Pearce, J. A. *et al. J. Volcan. geotherm. Res.* **44**, 189–229 (1990).
28. Woods, A. W. & Wohletz, K. *Nature* **350**, 225–227 (1991).
29. Stanley, D. J. & Sheng, H. *Nature* **320**, 733–735 (1985).
30. Jouanneau, J.-M. *Mar. Chem.* **21**, 189–197 (1987).
31. Murray, J. W., Top, Z. & Ozsoy, Z. *Deep-Sea Res.* **38**, suppl. 2A, S663–S689 (1991).
32. Boudreau, B. P. & Leblond, P. H. *Paleoceanography* **4**, 157–166 (1989).
33. Sullivan, D. G. *Nature* **333**, 552–554 (1988).
34. Honjo, S. *et al. Cruise Rep. R. V. Knorr 138–4, Leg 1 April 16–May 17, 1988; and Piri Reis Int. Contrib. Series No. 6.* (1988).

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Estimate of pyroclastic flow velocities resulting from explosive decompression of lava domes

Jonathan H. Fink & Susan W. Kieffer

Geology Department, Arizona State University, Tempe, Arizona 85287-1404, USA

APPARENTLY benign silicic domes or lava flows can travel for several kilometres and then suddenly collapse to generate pyroclastic phenomena capable of causing widespread destruction, as happened recently at Mount Unzen in Japan¹. Two sources have been proposed for the energy that propels such 'Peléan' or 'Merapi'-type² pyroclastic flows: gravitational collapse (supplemented by heating and expansion of air) and sudden expansion of pressurized gases from inside the lava flow. If gravity controls the energy transfer, then areas likely to be affected can be predicted on the basis of topography³, and the resulting deposits will bear a simple relationship to the part of the lava flow from which they issued. But if gas pressure adds a significant contribution, hazard assessment becomes more difficult because gas decompression adds velocities beyond those

acquired by gravitational forces, putting much larger areas at risk and forming pyroclastic deposits that are much more difficult to relate to their source. Here we estimate the initial velocities of pyroclastic flows generated by dome disintegration for a range of lava compositions and volatile contents, and offer a conceptual framework for correlating the dynamics of dome-front collapse with the resulting sediment record. Our results indicate that explosive decompression at distal portions of domes can cause velocities comparable to gravitational collapse, especially in cases where volatiles become locally concentrated above equilibrium values.

We focus on those relatively gas-poor ($[H_2O] < 1.0$ wt%) pyroclastic flows known as block-and-ash flows and associated surges, in contrast to the more gas-rich pumice and ash flows or ignimbrites that result from collapsing eruption columns. Although there are several detailed theoretical models for collapse of gassy eruption clouds directly over a primary eruptive vent⁴⁻⁶, and other studies containing direct and indirect observational evidence of lava dome disintegration^{7,8}, we are not aware of any quantitative models for the ways in which the front of a lava dome may transform itself into a pyroclastic flow. (Although lava domes and lava flows may be considered part of a morphological continuum, here for brevity we refer to both as lava domes.) Here we compare two endmember cases: (1) gravitational collapse of the front of a lava dome with initial velocities determined solely by conversion of potential energy into kinetic energy as pieces tumble to the ground^{9,10}; and (2) explosive emission of gas and rock from the flow front after exposure of lava at high pressure to ambient pressure by sudden removal of covering material^{7,11-13}. Gas-dynamic effects associated with endmember (2) can be large at early times, and it is this influence that we examine.

We rely on recent numerical models of large volcanic debris avalanches to estimate the maximum velocities due to gravity, and assume that the rheological and dynamic behaviour of collapsing domes and volcanic landslides will be similar because both are made up largely of hot blocks of lava. Calculated and observed velocities for the avalanches that flowed down the Toutle river valley at the onset of the 18 May 1980 eruption of Mount St Helens are typical of several other landslides¹⁴. For a range of rheological parameters, velocities were about 10 m s^{-1} for the first 10 s of flow, increasing up to 150 m s^{-1} at 30–60 s as the avalanche accelerated downslope. These values will be used below for comparison with decompression effects.

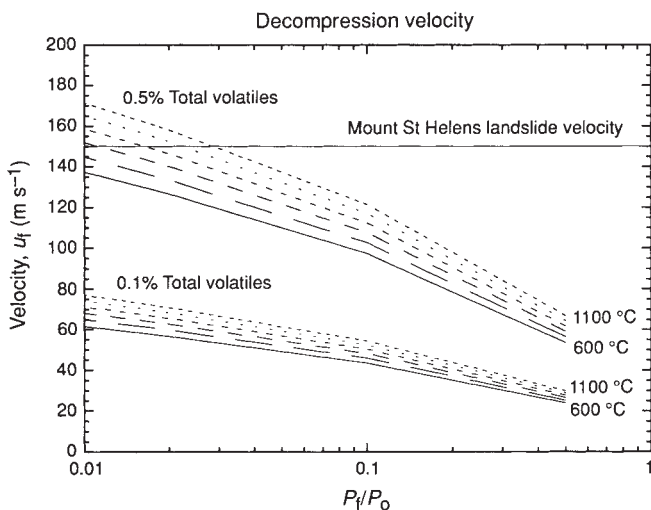


FIG.1 Plot of velocities of pyroclastic particles generated by collapse of lava dome fronts for a range of total volatile contents, internal pressures and temperatures. Highest values are comparable to those generated by gravitational collapse.

To first order, the violence of the emission of gas and rock from the front of a lava dome when covering material has been suddenly removed depends on the ratio of pressure of the gas within the dome (P_0 is the sum of lithostatic pressure and strength) to that in the atmosphere (P_i); the average initial temperature (T_0); and the gas to rock ratio (x). The problem is a difficult one of unsteady, multi-component (rock, water vapour, CO_2 and so on), multiphase (gas, liquid, solid), non-equilibrium (separated) flow. Because of limitations in available data, we used measured water content, but a more detailed analysis would use total exsolved gas. The analysis below ignores many of the complicating factors, but illustrates that the magnitude of the decompression effect can approach that due to gravity.

A minimum value for initial pressure in collapsing lava domes is the lithostatic load of a few bars to a few tens of bars, but internal pressure is also influenced by the strength of the lava. Although the upper surfaces of active lava domes appear blocky and fragmented, the inward increase in temperature means that there is some depth below which the lava remains molten and ductile¹⁵. Strengths of glassy obsidian measured in the laboratory¹⁶ are generally of the order of 0.1 MPa. Models for deformation of the Mount St Helens dacite dome^{17,18} yield estimates of the strength of the surface crust between about 0.1 and 10 MPa.

The amount of energy available during decompression also depends on the volatile content of the lava, which typically ranges from water values of 0.1 wt% for rhyolites to 0.4 for andesites¹⁹. Equally large variations can be found within a single extrusion, depending on emplacement sequence and vesicularity. At the Mount St Helens dacite dome, individual lobes had water contents that increased regularly from the flow front (typical values near 0.1 wt%) to the vent (0.3–0.4 wt%)²⁰. Dacite from the active Santiaguito dome in Guatemala has measured water contents ranging from 0.03 to 0.25 wt%²¹. In drill cores through one of the young Inyo rhyolite flows^{22,23} water contents ranged from 0.1 to 0.45 wt% with the highest concentrations found exclusively in glassy septa within coarsely vesicular zones located 15–20 m below the flow surface^{24,25}. These relations and petrographic evidence suggest that the water-rich zones result from migration and concentration of volatiles beneath a nondeforming carapace^{11,15} possibly supplemented by crystallization of anhydrous minerals in the flow interior¹².

Measured and estimated eruption temperatures for silicic lavas range between about 600 and $1,100^\circ\text{C}$ (ref.16). During advance of a lava dome, the surface temperature may drop to ambient levels but the interior, insulated by the blocky and vesicular carapace, should maintain temperatures near the eruption range for months to years^{26,27}.

From these data, in the calculations below we will assume the following bracketing values for properties of lava domes of rhyolite to andesite composition: $P_0 = 0.1\text{--}3.0$ MPa; $x = 0.1\text{--}0.5$ wt% and $T_0 = 600\text{--}1,100^\circ\text{C}$.

Our mathematical model for expansion of a pressurized lava dome involves many assumptions, of which the most severe are these. First, the expanding pyroclastic flow is considered to be homogeneous (in the fluid dynamical sense that there is no separation between the gas and the particles), and second, particle-particle interactions are neglected. The first assumption implies that initially the gas expansion 'instantaneously' accelerates the entrained particles up to the exit velocity, a limit approached only for particles much smaller than many of those found in block-and-ash flows. The second assumption is a serious restriction; at present there is no theoretical framework allowing rigorous consideration of 'granular' flow admixed with compressible gases. We estimate that these simplifications could cause the calculated velocities to exceed those obtained by fragments in a pyroclastic flow by a factor of two to three. Offsetting this effect, however, is the gas-phase separation at high velocities which will be discussed below.

Let us consider a volume of material at the front of the lava dome to be described by the thermodynamic parameters P_o , T_o , x (weight fraction of total exsolved volatiles, assumed here to be H_2O), and velocity u_o ($=0$ in the initial state). We assume that the flow front suddenly collapses and that the dusty gas mixture expands rapidly (adiabatically and isentropically) to a final pressure P_f (taken to be atmospheric pressure, 0.1 MPa). The process is further constrained to be one-dimensional, isothermal and frictionless, and the initial pressure P_o is assumed to be at least two to three times atmospheric.

We calculate the final velocity, u_f , by deriving an equation of state and an adiabatic expansion law for the dense mixture. The method of solution follows that for an inviscid pseudo-gas²⁸. Our derivation demonstrates that thermodynamically the mixture can be treated as a perfect gas with the gas constant, R , modified to account for the composition of the gas phase(s) and the mass-loading of solids; with the isentropic exponent γ , modified to account for the heat transfer; and with the volume modified to account for the presence of the solid phase. The quantity of interest is the velocity obtained on decompression, given by the St Venant–Wantzel equation:

$$u_f = \{2\gamma RT[(1 - P_f/P_o)/(\gamma - 1)]\}^{1/2}$$

The equation gives the maximum speed attainable by a pseudo-gas that expands isentropically from an infinite reservoir²⁹. In a real gas, friction reduces the obtainable velocity.

For H₂O vapour, $R_{\text{H}_2\text{O}}$ is $0.46 \times 10^7 \text{ erg K}^{-1} \text{ g}^{-1}$, C_p is $4.19 \text{ J g}^{-1} \text{ K}^{-1}$, and C_v is $3.14 \text{ J g}^{-1} \text{ K}^{-1}$. For typical solid fragments, the specific heat capacity $C_s = 1.00 \text{ J g}^{-1} \text{ K}^{-1}$. Using these values, and for $x=0.1\text{--}0.5\%$; $T=600\text{--}1,100^\circ\text{C}$; and pressure ratios $P_d/P_o = 0.03\text{--}1$, we derive the values of plausible velocity shown in Fig. 1. The maximum calculated excess velocity for these conditions is 170 m s^{-1} for a lava at an eruption temperature of $1,100^\circ\text{C}$, water content of $0.5 \text{ wt}\%$, and pressure of 100 bar within the lava dome. Even for more conservative values for a rhyolite flow of 800°C , $0.4 \text{ wt}\% \text{ H}_2\text{O}$, and 10 bar pressure, an excess velocity of over 100 m s^{-1} is produced.

Despite uncertainties in the model, it is clear that gas pressure is capable of propelling pyroclastic material to velocities comparable to those for gravity alone, and the gas–dust fraction may travel even faster. Because gas at high pressures expands laterally on decompression, the area over which such high velocities would occur will be broader and more difficult to

predict than those affected by gravitational collapse alone. If gassy blocks tumble from the front of a flow or are crushed in transit, local high-velocity gas emissions may also occur⁸.

Much of our knowledge of these endogenic explosive phenomena must be gleaned from analysis of the resulting pyroclastic deposits. If simple gravitational collapse occurs, deposits should resemble landslide deposits in texture and be confined to areas limited by potential energy conversion and energy dissipation. In contrast, gas-driven decompression may produce a variety of deposits, and can be driven outwards or upwards beyond gravitationally determined limits by conversion of stored enthalpy to kinetic energy¹³. Some ideas about the relation between gas decompression and resulting deposits are shown in Fig. 2.

The front of a dome is assumed to fall off, for example by oversteepening, at $x=0$, $t=0$ (upper left of Fig. 2). As shown in the main part of the figure, this sudden exposure of the dome's interior to ambient pressure results in a complex decompression wave which would be called a rarefaction wave in simple gas dynamics. However, this decompression is probably much more complex in dome failure with expansion and acceleration of free volatile phases, nucleation and growth of dissolved volatiles if kinetics permit, and fragmentation of the liquid or semi-rigid component.

In our calculations, the acceleration of particles and gas is assumed to be homogeneous, but as shown in the figure, there is probably substantial segregation of the gas phase, the fines and the coarse particles. The segregation process could produce different flow regimes (ash hurricanes, surges, block and ash flows) and a variety of depositional facies depending on processes downslope as well as on the initial flow segregation near the dome. The relations may not be simple, depending on relative velocities of the different flow regimes as well as on such factors as slope, channel formation and external water.

In summary, differences in pressure and gas content within lava domes can produce variations in the intensity of pyroclastic phenomena generated by flow-front collapse. Estimating the magnitude of explosive effects thus requires knowledge of the variability of front thickness and lava volatile contents. Increases in lava vesicularity (usually detectable on aerial photographs as a darkening of the lava surface or ballistic expulsion of blocks from the flow surface) may reflect magma volatile content and can serve as warning signs of an enhanced likelihood of explosive decompression. The course of future pyroclastic flows cannot be reliably determined solely by tracing the paths of prior flows generated by collapse of any given lava-dome lobe, nor by noting the elevation of the flow front relative to surrounding topography. In particular, mapped distributions of older block-and-ash deposits probably greatly underestimate the potential devastation zone because they exclude evidence of the less well-preserved gassy parts of previous flows. On the other hand, calculated estimates of decompression velocities can help delineate zones likely to be affected by future volcanic blasts and surges.

Mount Unzen's pyroclastic flow of 3 June 1991, which killed three volcanologists and 40 others, illustrates how poorly the variability of dome processes has been appreciated, even among experts. If this exceptionally destructive flow emanated from a portion of the dome that was even slightly more volatile-enriched, it could have had a considerably higher initial velocity, more vertically extensive ash hurricane cloud, longer runout distance and broader area of impact. Although we will never know, monitoring of the dome surface might have revealed changes in lava texture or fumarole activity and helped to avert the tragedy. □

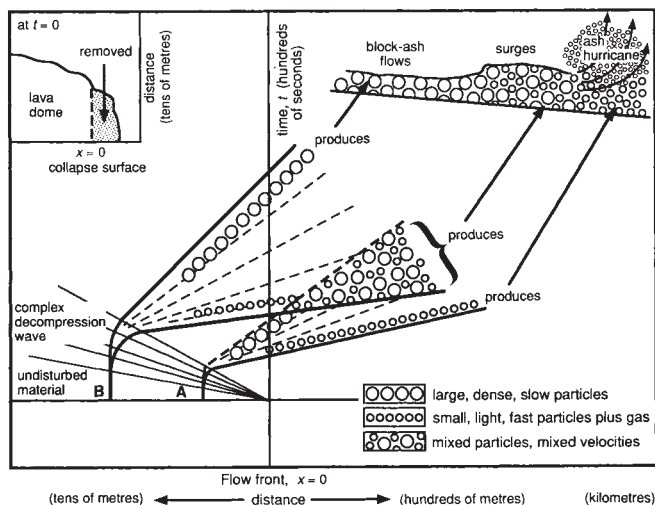


FIG.2 Schematic space-time diagram of the evolution of dome collapse and flow initiation. The slopes of lines radiating from the origin represent relative velocities. The collapse surface migrates from origin A to B.

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1. Nakada, S. Fujii, T. J. *Volcan. geotherm. Res.* **54**, 319–334 (1993).
2. Macdonald, G. A. *Volcanoes* (Prentice Hall, Englewood Cliffs, New Jersey, 1972).
3. Malin, M. C. & Sheridan, M. F. *Science* **217**, 637–639 (1982).
4. Smith, R. L. *Geol. Soc. Am. Bull.* **71**, 795–842 (1960).

5. Sparks, R. S. J., Wilson, L. & Hulme, G. *J. geophys. Res.* **83**, 1727–1739 (1978).
6. Woods, A. W. & Caulfield, C. P. *J. geophys. Res.* **97**, 6699–6712 (1992).
7. Rose, W. I. Jr, Pearson, T. & Bonis, S. *Bull. Volcanol.* **40**, 53–70 (1976).
8. Sato, H., Fujii, T. & Nakada, S. *Nature* **360**, 664–666 (1992).
9. Stith, J. L., Hobbs, P. V. & Radke, L. F. *Geophys. Res. Lett.* **4**, 259–262 (1977).
10. Mellors, R. A., Waitt, R. B. & Swanson, D. A. *Bull. Volcanol.* **50**, 14–25 (1988).
11. Fink, J. H. & Manley, C. R. *IAVCEI Proc. Volcanol.* **1**, 169–179 (1989).
12. Bardintzeff, J. M. *J. Geodynam.* **3**, 303–325 (1985).
13. Kieffer, S. W. U. S. *Geol. Surv. Prof. Pap.* **1250**, 379–400 (1982).
14. Sousa, J. & Voight, B. *Geotechnique* **41** (4), 515–538 (1991).
15. Fink, J. H. & Manley, C. R. *Geol. Soc. Am. spec. Pap.* **212**, 77–88 (1987).
16. McBirney, A. R. & Murase, T. A. *Rev. Earth planet. Sci.* **12**, 337–357 (1984).
17. Griffiths, R. W. & Fink, J. H. *J. Fluid Mech.* **252**, 667–702 (1993).
18. Iverson, R. *IAVCEI Proc. Volcanol.* **2**, 47–69 (1990).
19. Williams, H. & McBirney, A. R. *Volcanology* (Freeman Cooper, San Francisco, 1979).
20. Anderson, S. W. & Fink, J. H. *IAVCEI Proc. Volcanol.* **2**, 25–46 (1990).
21. Anderson, S. W., Fink, J. H., & Rose, W. I. *Jr Eos* **71**, 1720 (1990).
22. Eichelberger, J. C., Carrigan, C. R., Westrich, H. R. & Price, R. H. *Nature* **323**, 598–602 (1986).
23. Manley, C. R. & Fink, J. H. *Geology* **15**, 549–552 (1987).
24. Westrich, H. R., Stockman, H. W. & Eichelberger, J. C. *J. geophys. Res.* **93**, 6503–6511 (1988).
25. Westrich, H. R. & Eichelberger, J. C. *N. M. Bur. Mines Min. Res. Bull.* **131**, 206–291 (1989).
26. Manley, C. R. *J. Volcan. geotherm. Res.* **18**, 224–233 (1992).
27. Dzurisin, D., Denlinger, R. P. & Rosenbaum, J. G. *J. geophys. Res.* **95**, 2763–2780 (1990).
28. Wallis, G. B. *One-dimensional Two-Phase Flow* (McGraw Hill, New York, 1969).
29. Zucrow, M. J. & Hoffman, J. D. *Gas Dynamics*, Vol. 1 (Wiley, New York, 1976).

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Evidence for a K/T impact event in the Pacific Ocean

E. Robin, L. Froget, C. Jéhanno & R. Rocchla

Centre des Faibles Radioactivités CEA/CNRS, 91190 Gif-sur-Yvette, France

THE chemical, mineralogical and isotopic characteristics of deposits at the Cretaceous/Tertiary (K/T) boundary are suggestive of a large impact event, the prime candidate¹ being the Chicxulub crater in Yucatan, Mexico. Spinel-bearing spherules, which may be associated with such impacts, have been reported² at several K/T boundary sites worldwide, but their origin is still uncertain. We have examined the spinel-bearing material recovered from K/T boundary deposits at site 577 in the Pacific Ocean³ and find two distinct populations of particles: spherules with dendritic spinel textures dispersed throughout the grains and irregularly shaped fragments with spinels essentially confined to the rim. The morphology and composition of the particles are characteristic of melted and partially melted meteoritic ablation debris, but their location is difficult to reconcile with an impact on the Yucatan peninsula, some 10,000 km away. We suggest instead that the spinel-bearing particles at site 577 are derived from the impact of a 2-km asteroid in the Pacific Ocean, and that several accretionary events of this type are required to explain the global distribution of spinel-bearing spherules at the K/T boundary.

At Deep Sea Drilling Program (DSDP) site 577 (32° 26.51' N and 157° 43.40' E, on the flank of the Shatsky rise³) a complete K/T boundary transition is recorded in a nearly pure carbonate (>95%) sequence^{3,4}. The palaeontological boundary here, defined by nanofossil studies⁵, coincides with an Ir anomaly⁶ and with the occurrence of spinel-bearing spheroids^{2,4}.

We have looked for such spheroids and other debris at hole 577B-1, in a 523-mg sample collected at level 577B-1-4, 69–71 cm deep⁶. The carbonate fraction was dissolved in 10% acetic acid and the coarse fraction (> 50 µm), weighing ~ 4.2 mg, was irradiated for 72 hours in a flux of 2.3×10^{14} neutrons cm⁻² s⁻¹ from the OSIRIS reactor at Pierre SUE Laboratory (Saclay). The fine fraction (<50 µm), weighing ~ 25.8 mg, and 100 mg of

TABLE 1 Electron microprobe analyses

Oxides (%)	Smectite	Spinel			
		1	2	3	4
Na ₂ O	<0.5	—	—	—	—
MgO	4.8 (0.6)	19.5	20.2	16.0	15.0
Al ₂ O ₃	10.3 (1.1)	22.8	12.0	5.3	3.8
SiO ₂	56.6 (1.8)	0.6	1.7	0.7	1.5
K ₂ O	2.7 (0.9)	—	—	—	—
CaO	2.7 (0.6)	0.4	0.7	0.8	0.9
TiO ₂	0.5 (0.1)	0.3	0.6	0.2	<0.1
Cr ₂ O ₃	<0.1	0.2	0.2	1.4	1.0
MnO	<0.1	0.4	0.7	0.8	0.7
FeO	—	3.9	0.6	2.4	0.6
Fe ₂ O ₃	21.5 (1.9)	51.4	59.5	68.5	69.0
NiO	<0.2	1.5	2.5	4.9	6.9
Sum	100.0	101.0	98.7	101.0	99.5

Electron microprobe analyses (in weight %) of the clay matrix (smectite) and spinel crystals in spheroids and fragments recovered from hole 577B-1. The smectite composition is given from the analysis of 30 particles; values in parentheses represent 1σ standard deviation. Most of the spinels (>80%) have composition corresponding to analyses 1 and 2. However, compositions 3 and 4 are also observed. FeO and Fe₂O₃ are calculated assuming the spinels are stoichiometric.

whole-rock sample were also irradiated for 2 hours. These samples and about 100 particles randomly extracted from the coarse fraction were analysed for Ir, Ni, Co, Cr, Sc and rare-earth elements with a high-purity Ge γ-ray detector. Individual particles were then mounted in epoxy and polished for detailed scanning electron microscopy and electron microprobe analysis.

Particles from the coarse fraction were sorted in two populations according to morphology: spherules (Fig. 1a and b, 80–90% of the particles) and irregular fragments (Fig. 1d and e, 10–20% of the particles). The spherules from population 1 contain varying amounts of spinels embedded in a clay matrix. The dendritic and skeletal morphologies of the spinels and their uniform distribution throughout the grains are indicative of rapid crystallization from completely melted droplets. The irregular fragments from population 2 are composed of nearly pure clay rimmed with tiny spinels. The existence of a continuous rim of spinels surrounding a core relatively free of these crystals shows that these particles are not parts of broken spherules from population 1, or part of other debris, but are undamaged whole particles which have undergone partial melting. Similar features are observed in cosmic objects such as micrometeorites^{7–12} and ablation debris from natural and artificial meteorites^{13–15} which have been heated and oxidized in the Earth's atmosphere (Fig. 1c, f).

X-ray diffraction analysis of a set of spherules indicates that the dominant spinel endmember is magnesioferrite, consistent with the high ferric/ferrous ratio derived from compositional data ($\text{Fe}^{3+}/\text{Fe}^{2+} > 10$; Table 1). Our chemical analyses of about 100 spinels are consistent with those previously reported¹⁶: they have high Ni (> 1%) contents, low Ti contents (< 1%) and a high iron oxidation state ($\text{Fe}^{3+}/\text{Fe total} \approx 99$ at %, Table 1). The only process known to form spinels with such a composition is ablation and oxidation of meteoritic debris in the atmosphere^{17–20}. Such spinels have also been found in spherules associated with oceanic impact debris from Late Pliocene sediments²¹. The existence of such spinels in nearly all the particles at site 577 supports the view that they mostly derive from ablation in the atmosphere of meteoritic material. As

already noted by Kyte and Smit¹⁶, most of the spinels in hole 577B-1 have high average Al and Fe³⁺ contents. In this they are similar to K/T spinels from North Pacific GPC3 (30° 19.9' N, 157° 49.4' W)¹⁶ and South Pacific DSDP site 596 (23° 51.20' S, 165° 39.27' W)²², but are significantly different from those found at other K/T sites^{16,23-27}. This implies that spinel-bearing debris from the Pacific Ocean all has a common and local origin. X-ray diffraction analyses also show that the clay matrix is smectite (Table 1), and not glauconite as previously reported². Smectite results from the alteration of minerals less resistant than spinels, presumably olivine which is often associated with spinels in ablation particles⁷⁻¹⁵. Although these minerals are now replaced by smectite, their original morphology has been preserved and is visible on Fig. 1a, b and e.

In particles from population 1, Ir contents range from less than 1.5 ng g⁻¹ up to 610 ng g⁻¹, with an average value of 84 ng g⁻¹ (86 spherules analysed so far; Table 2). This wide range of Ir compositions, with only few spherules having chondritic Ir abundances, is consistent with the range of Ir contents observed in cosmic ablation spheres⁹⁻¹¹ (Fig. 2). In particles from population 2 (11 particles), Ir contents range from 95 ng g⁻¹ to 1,200 ng g⁻¹, with an average value of 530 ng g⁻¹, very close to the Ir abundance of C1 chondrites. Such high Ir contents in individual spinel-bearing particles have been found only in chondritic ablation debris⁹⁻¹². This is the most compelling evidence that pure meteorite debris is deposited at site 577. Additional arguments are the systematic Cr, Co and Ni enrichments in spheroids and fragments, with abundances sometimes approaching those in meteorites (Table 2), and the marked depletion of REEs compared with their concentration in the sediment (in whole-rock samples and in acetic-leached decarbonated samples; Table 2). The markedly low REE contents also rule out mixing with more than 10% of terrestrial material, thus confirming that even spheroids with low Ir content derive from nearly pure meteorite debris. The slight but systematic enrichment of REEs relative to chondrite, with light REEs slightly enriched relative to heavy REEs, may be attributed to postdepositional contamination by the surrounding REE-rich sediment.

As all particles in the 4.2-mg coarse fraction fall into either population 1 or 2, the concentration of ablation debris > 50 µm in the 523 mg sample is therefore ~ 8 mg g⁻¹, accounting for 20% of the total Ir in that sample. This is a minimum value

TABLE 2 INAA analyses

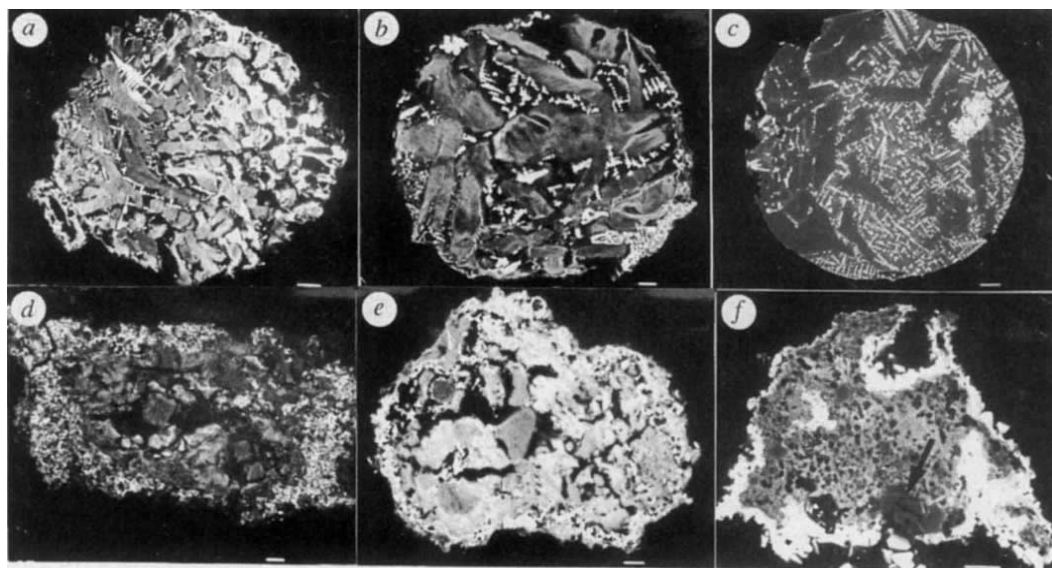
	Sediment			Population 1		Population 2	
	WRS	FF	CF	Ir-rich spherules		Fragments	
				Average	Range	Average	Range
Sc	2.4	17.5	26.4	21.2	9.4-32.6	20.00	13.1-39.4
Cr	13.5	125	665	920	30.4-200	1,600	600-3,600
Fe%	0.5	10.0	20.0	22.0	15.0-39.0	18.8	13.4-32.9
Co	4.7	14	85	167	2-500	140	30-340
Ni	20.0	100	1,300	2,100	60-9,000	3,200	500-11,000
La	23.8	200	65.0	4.4	<2.0-8.5	3.6	1.1-3.2
Ce	5.9	41	17.2	4.9	1.1-13.4	8.4	<6-8.4
Nd	14.1	130	49.2	6.1	<2.0-20	<12.0	0.65-2.8
Sm	3.9	42	24.8	1.6	<0.6-3.3	1.7	0.85-1.70
Eu	1.0	10.4	5.4	0.54	0.15-1.60	1.3	-
Tb	0.6	5.9	4.0	0.38	0.08-0.96	<0.75	-
Yb	2.40	14	9.0	1.20	<0.36-3.5	<1.45	-
Lu	0.29	0.94	0.9	0.08	0.03-0.16	<0.15	95-1200
Ir(p.p.b.)	5.3	70	115	84	<1.5-610	530	-

Elemental contents determined by instrumental neutron activation analysis (INAA) for whole-rock sample 577B-1-4, 69-71 cm (WRS), the fine (< 50 µm) and coarse (> 50 µm) fraction (respectively FF and CF) from the decarbonated sediment, as well as for 97 individual spinel-bearing debris (86 spherules and 11 fragments) randomly extracted from the coarse fraction. Note that the average Ir content of the coarse fraction (~ 115 ng g⁻¹) and of populations 1 and 2 (~ 130 ng g⁻¹) are consistent with each other, indicating that the extraction procedure has not introduced any selection effect. Concentrations are in µg g⁻¹ except Ir which is in ng g⁻¹. Typical 1σ standard deviation is < 5% for Cr, Sc, Co and Ir; 5-25% for Ni and REE. Our bulk sediment analysis is in good agreement with previous data⁶.

because it does not include any contribution from the fine fraction. Assuming that in this fraction Ir is carried by the same populations of particles as in the coarse fraction, we can infer that the concentration of ablation debris could be as high as ~ 30 mg g⁻¹, accounting for 80% of the total Ir.

The total infall of ablation debris in hole 577B-1 cannot be directly determined from our data alone, as we have analysed only one sample from this site. However, a reasonable estimate may be inferred from observations in the nearby hole 577

FIG. 1 Set of scanning electron microscope images in the backscattered mode of polished sections of H577B-1 particles and of two cosmic ablation debris. Scale bar 10 µm. a, b, Typical spinel-bearing spheroids from hole 577B-1 with dendritic spinels (white spots) present in the groundmass and smectitic phases (presumably olivine before alteration) having preserved crystal morphologies (dark areas); these spherules contain respectively 610 and 520 ng g⁻¹ of Ir. c, A natural cosmic ablation sphere from Greenland cryoconites⁹ with comparable morphological, mineralogical and chemical features: white spots are spinels and dark areas are recrystallized olivine and glass. d, e, Irregular fragments indicating partial melting with tiny spinels present in a continuous rim; they contain,



respectively, 1,200 and 335 ng g⁻¹ of Ir. f, Partially melted debris from laboratory experiment on Murray chondrite showing a spinel rim and relic olivine crystals (arrows).

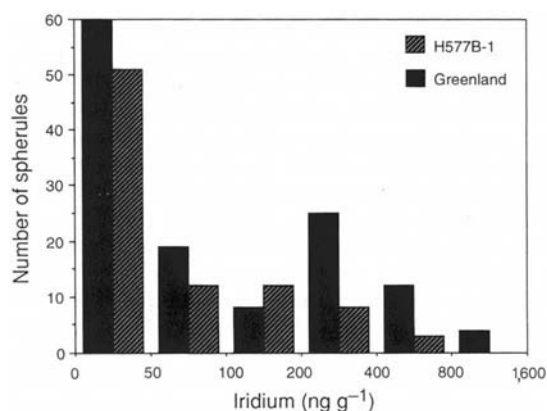


FIG. 2 Histogram showing the range of Ir compositions in H577B-1 spherules. For comparison, the range of Ir compositions of natural cosmic ablation spheres from Greenland cryoconites⁹ are plotted (data are from ref. 10).

(ref. 4). At this site, the stratigraphic distribution of the spinel-bearing spheroids is characterized by a rapid rise followed by a nearly exponentially decreasing tail ($C_m \exp(-z/d)$ where C_m is the maximum concentration and z is the sample position in cm, with $d=4$ cm). Assuming a similar distribution at hole 577B-1, a dry sediment density²⁸ ≈ 1.15 g cm⁻³ and a maximum concentration C_m ranging from 8 to 30 mg g⁻¹, we obtain a flux $C_m \rho d \approx 40$ –140 mg cm⁻². Such a flux cannot be explained by the accretion of particles from the steady-state flux of micrometeoroids because it would require accumulation for 10–40 million years (the present micrometeoroid flux on Earth²⁹ is 3×10^{-6} mg cm⁻² yr⁻¹). Only an exceptional accretionary event could explain our observations.

It is now widely perceived that the presumed Chicxulub crater is responsible for the K/T event¹ although an impact in this region is still controversial²⁶. It is difficult, in any case, to reconcile our finding of ablation debris with an impact of a 10-km asteroid on the Yucatan peninsula which is $\sim 10,000$ km away from site 577. Only melted or condensed spherules of mixed projectile and target material, ejected on ballistic orbits, can be transported over such distances³⁰; the irregular particles from population 2, which are obviously only partially melted, cannot result from such an event. The survival and preservation of significant amounts of pure meteoritic material are unlikely in a cratering event on land, because the entire projectile and a large fraction of the target are volatilized³¹. In addition, the amount of meteoritic material is always low in impact-melt rocks (in general $\sim 10^{-2}$ relative to C1-chondrites³²), and still lower in the long-range ejecta ($\sim 10^{-3}$ – 10^{-5})^{33,34}. Spinel-bearing spherules cannot be confused with microtektites, which are extremely reduced objects free of any crystalline inclusion^{33–35}.

The most probable source for the spinel-bearing debris at site 577 is atmospheric ablation of nearly pure meteoritic debris generated by an impact in the Pacific Ocean. Several lines of evidence support this view: first, Al-Fe³⁺-rich spinels are restricted to the Pacific Ocean, which can only be explained by an impact in that region; second, large amount of pure meteoritic debris can survive in an oceanic impact³⁶; third, impact models predict that material of dominantly meteoritic composition can be dispersed over a large area following an impact in the ocean³⁷, especially for low-angle impacts³⁸; and finally, highly oxidized spinels are formed in such events²¹.

Although the geographical dispersion of the ablation debris is unknown, an estimate of the diameter of the presumed Pacific impactor may be derived from the geographical dispersion of the Al-Fe³⁺-rich spinels, assuming that the composition of these spinels is specific of that impact. Al-Fe³⁺-rich spinels have been reported in K/T boundary sediments at GPC3¹⁶ and at South Pacific DSDP site 596²², which are located $\sim 4,000$ – $6,000$ km away from site 577. Considering a maximum dispersion area of $\sim 2 \times 10^7$ km², a constant flux of ~ 40 – 140 mg cm⁻² over this area and a projectile density of 3 g cm⁻³, we can estimate that the initial bolide diameter was ~ 2 km.

We have considered here only the spinels from the Pacific Ocean, because they all have the same average composition and can therefore be assumed to derive from the same source. However, spinels of quite different compositions have been reported worldwide at the K/T boundary, in sections located in Italy^{16,23,24}, Spain^{24,25}, New Zealand²⁵, Haiti²⁶ and in deep-sea cores in the Pacific¹⁶, Atlantic¹⁶ and Indian oceans²⁷. From this study, we can reasonably conclude that K/T spinels from all sites were originally contained in ablation debris deriving from pure meteoritic material. If so, the strong variation in spinel compositions observed from site to site^{16,19} could best be explained by multiple accretionary events³⁹, rather than by a single large impact. □

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- Sharpton, V.L. et al. *Nature* **359**, 819–821 (1992).
- Smit, J. & Romaine, A.J.T. *Earth planet. Sci. Lett.* **74**, 155–170 (1985).
- Heath, G.R. et al. *Init. Rep. DSDP 86*, Site 577 (U.S. Govt Printing Office, Washington DC, 1985).
- Gerstel, J., Thunell, R.C., Zachos, J.C. & Arthur, M.A. *Paleoceanography* **1**, 97–117 (1986).
- Monechi, S. *Init. Rep. DSDP 86*, 301–336 (1985).
- Michel, H.V., Asaro, F., Alvarez, W. & Alvarez, L.W. *Init. Rep. DSDP 86*, 533–538 (1985).
- Blanchard, M.B., Brownlee, D.E., Bunch, T.E., Hodge, P.W. & Kyte, F.T. *Earth planet. Sci. Lett.* **46**, 178–190 (1980).
- Brownlee, D.E. in *The Sea* (ed. Emiliani, C.) Vol. **7**, 723–762 (Wiley, New York, 1981).
- Maurette, M., Jéhanno, C., Robin, E. & Hammer, C. *Nature* **328**, 699–720 (1987).
- Robin, E. thesis, Univ. Paris XI at Orsay (1988).
- Koeberl, C. & Hagen, E. *Geochim. cosmochim. Acta* **53**, 937–944 (1989).
- Robin, E., Christophe Michel-Levy, N., Bourou-Denise, M. & Jéhanno, C. *Earth planet. Sci. Lett.* **97**, 162–176 (1990).
- Blanchard, M.B., Cunningham, G. & Brownlee, D.E. *Meteoritics* **9**, 316–317 (1974).
- Blanchard, M.B. & Cunningham, G. *J. geophys. Res.* **79**, 3973–3980 (1974).
- Brownlee, D.E., Blanchard, M.B., Cunningham, G.C., Beauchamp, R.H. & Fruland, R.J. *geophys. Res.* **80**, 4917–4924 (1975).
- Kyte, F.T. & Smit, J. *Geology* **14**, 485–487 (1986).
- Robin, E., Bonté, Ph., Froget, L., Jéhanno, C. & Rocchia R. *Earth planet. Sci. Lett.* **108**, 181–190 (1992).
- Robin, E., Bonté, Ph., Froget, L., Jéhanno, C. & Rocchia R. *23rd lunar planet. Sci. Conf. (Abstr.)*, 1159–1160 (1992).
- Robin, E., Bonté, Ph., Froget, L., Jéhanno, C. & Rocchia R. *23rd lunar planet. Sci. Conf. (Abstr.)*, 1161–1162 (1992).
- Jéhanno, C., Boclet, D., Bonté, Ph., Castellarin, A. & Rocchia R. *Proc. lunar planet. Sci. Conf.* **18**, 623–630 (1988).
- Margolis, S.V., Claeys, Ph. & Kyte, F.T. *Science* **251**, 1594–1597 (1991).
- Kyte, F.T., Zhou, L. & Bohor, B.F. *Meteoritics* **26**, 361 (1991).
- Smit, J. & Kyte, F.T. *Nature* **310**, 403–405 (1984).
- Jéhanno, C., Boclet, D., Bonté, Ph., Devineau, J. & Rocchia, R. *Mém. Soc. géol. France* **150**, 81–94 (1987).
- Bohor, B.F., Foord, E.E. & Ganapathy, R. *Earth planet. Sci. Lett.* **81**, 57–66 (1986).
- Jéhanno, C. et al. *Earth planet. Sci. Lett.* **109**, 229–241 (1992).
- Rocchia, R. et al. *Proc. ODP 122*, 753–762 (1992).
- Zachos, J.C., Arthur, M.A., Thunell, R.C., Williams, D.F. & Tappa, E.J. *Init. Rep. DSDP 86*, 513–528 (1985).
- Grün, E., Zook, H.A., Fechtig, H. & Giese, R.H. *Icarus* **62**, 244–272 (1985).
- Melosh, H.J. & Vickery, A.M. *Nature* **350**, 494–497 (1991).
- Melosh, H.J. *A. Rev. Earth planet. Sci.* **8**, 65–93 (1980).
- Grieve, R.A.F. *Geol. Soc. Am. spec. Pap.* **190**, 25–37 (1982).
- Glass, B.P. *Tectonophysics* **171**, 393–404 (1990).
- Koeberl, C. *Tectonophysics* **171**, 405–422 (1990).
- Fudali, R.F. *Geochim. cosmochim. Acta* **51**, 2749–2756 (1987).
- Kyte, F.T. & Brownlee, D.E. *Geochim. cosmochim. Acta* **49**, 1095–1108 (1985).
- Melosh, H.J. *Geol. Soc. Am. spec. Pap.* **190**, 121–127 (1982).
- Schultz, P.H. & Gault, D.E. *Geol. Soc. Am. spec. Pap.* **247**, 239–261 (1990).
- Kyte, F.T., Zhou, Z. & Watson, J.T. *Nature* **288**, 651–656 (1980).

Disruptive selection and the genetic basis of bill size polymorphism in the African finch *Pyrenestes*

Thomas Bates Smith

Department of Biology, San Francisco State University,
1600 Holloway Avenue, San Francisco, California 94132, USA

MECHANISMS producing and maintaining discrete polymorphisms have long fascinated evolutionary biologists^{1,2}. Despite the ubiquity of non-sex-limited polymorphisms in vertebrates, the evolutionary factors maintaining them are well understood in only a few instances³. The African finch *Pyrenestes* is unique among birds in exhibiting a non-sex-determined polymorphism in bill size^{4,5}. Morphs breed randomly with respect to bill size and differ in diet and feeding performance on soft and hard seeds^{4,6}. I present here: (1) new data showing that the polymorphism appears to result from a single genetic factor; (2) support from long-term field studies for earlier suggestions that disruptive selection is acting on bill size; and (3) data revealing the presence of a possible third, much larger morph. Results suggest that the polymorphism may have arisen through single mutations, where morphs occupy distinct adaptive peaks through differences in feeding performance on seeds differing in hardness.

I conducted field work for three years between 1983 and 1990 on the black-bellied seedcracker (*P. o. ostrinus*) in Cameroon. Over 2,700 individuals were netted, banded and measured⁴. Evidence of natural selection on six morphological characters was examined over a 7-year period (Fig. 1). Because annual variation in rainfall and seed production was low^{6,7}, I assume the direction and intensity of selection varied little across years. I estimated selection over the entire study, rather than in each

year or across a single season, to maximize sample size. Previous research suggested that selection primarily takes place during the major dry season (February–May), during which food is least abundant and inter- and intraspecific competition is most intense⁷. Analysis centred on survival of juveniles because selection tends to be most intense on this age class⁸. Juveniles of either sex measured and released between 1983–1986 were compared with those individuals recaptured during an 8-month period in 1989 and 1990. I estimated natural selection on each of six morphological characters using the cubic spline technique⁹, which is a nonparametric technique that provides a quantitative prediction of survival probabilities across a range of differing character sizes, allowing an estimate of fitness for a particular character value, and a fitness surface for the character in the population. I found evidence of disruptive selection occurring on four of the six characters: lower mandible width, lower mandible length, upper mandible depth and tarsus length (Fig. 1). In each case there is evidence of a bimodal fitness surface with two peaks and a valley between them. No selection was detected on wing or upper mandible length.

Disruptive selection is probably related to seed quality. Bill size, particularly lower mandible width, is the most important character in predicting the time taken to crack seeds⁴ and has been shown to be the primary target of selection⁸. Morphs feed primarily on seeds of two species of sedge; these seeds are similar in size but differ dramatically in hardness. Large morphs feed more efficiently on the hard-seeded sedge (*Scleria verrucosa*, mean hardness = 153 Newtons, s.e. ± 2.0); small morphs feed more efficiently on the soft seeded sedge (*S. goossensii*, mean hardness = 13 N, s.e. ± 2.0)⁶. Despite evidence of disruptive selection on lower mandible length and tarsus, neither of these characters shows a bimodal distribution, in contrast to the other two characters on which selection occurred. This may suggest that components of fitness other than juvenile to adult survival may be acting on these characters.

In addition, during 1985 and 1989, 97 finches were exported from my main study site in south-central Cameroon (03°45' N,

FIG. 1 *a*, Fitness surfaces of the four characters showing evidence of natural selection calculated using the cubic spline technique. Splines were estimated for each character (solid lines), with confidence limits for each spline (dashed lines) generated by bootstrapping in which the original data set was resampled 100 times⁹. Shaded histograms (*b*) show distributions of juveniles that did not survive, black histograms those that survived. *c*, Distribution of characters in the adult population. Juveniles that were not captured in 1989–90 were assigned an absolute fitness of 0, and those recaptured, a value of 1. Only juveniles that were 2–3 months old and had already reached adult size⁵ were used in the analysis. Sampling adjacent regions to the study site revealed no evidence of differential dispersal of particular phenotypes. Because splines are not designed to handle characters with major gaps in their distributions (D. Schluter, personal communication), splines were calculated for each morph separately for lower mandible width. Additionally, because the performance of the bootstrap to estimate confidence limits has not yet been tested through simulation, bootstrap results should be interpreted cautiously⁹.

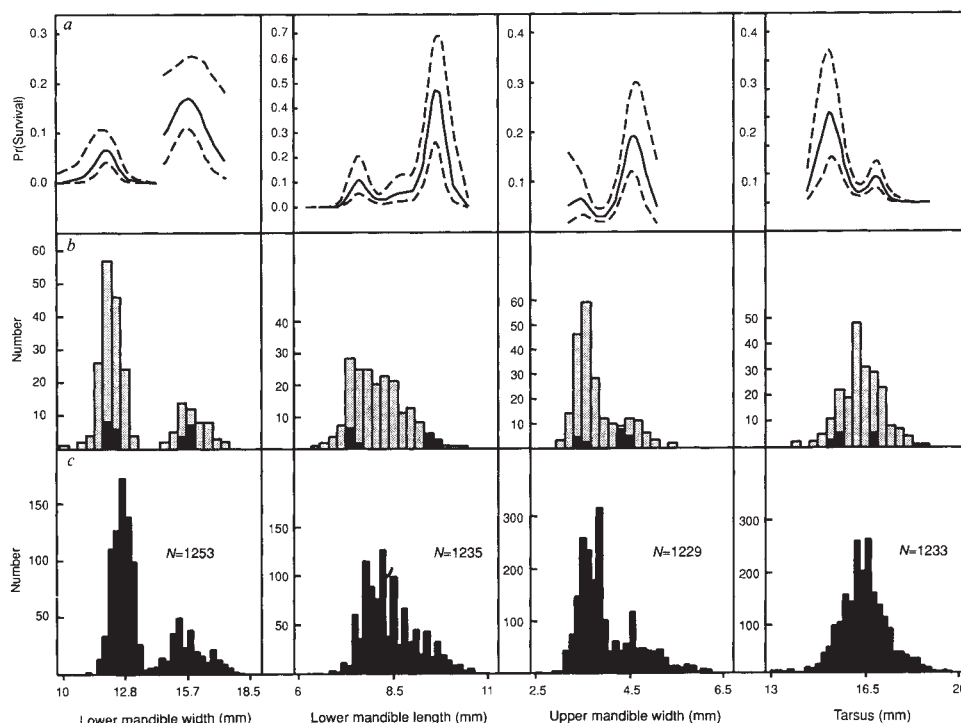
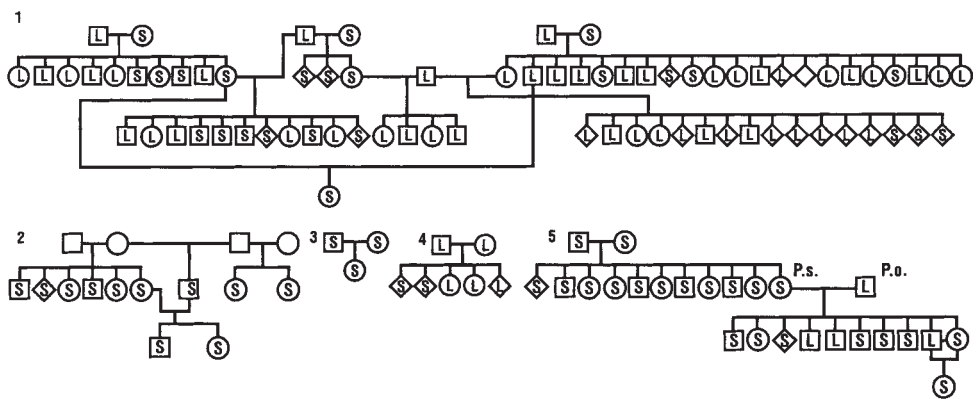


FIG. 2 Pedigrees showing matings between small (S)- and large (L)-billed morphs (lower mandible width <14 mm and >14 mm, respectively). Squares and circles represent adult males and females, respectively, triangles are juveniles less than 6 months old. Bill size was determined on the basis of lower mandible width which reaches adult size at four months of age⁵. Individuals in which bill size was not measured or in cases of juveniles which died before bill size could be determined are blank. Pedigrees 1 and 4 represent matings involving the race *P. o. ostrinus* (P.o.), matings 2 and 3 the race *P. o. sanguineus* (P.s.) and within pedigree 5 a cross between *P. o. sanguineus* and *P. o. ostrinus*. Heritabilities and repeatabilities for 6 morphological characters from 11 families and 69 offspring were calculated from regressions of offspring on mid-parents after data was $\log_e$ transformed¹⁷. Only juveniles that had reached adult size were used in the analysis. Captive bred (and wild) nestlings reach near-adult size while being fed by their parents and do not crack seeds until after fledging. Thus, there is no opportunity for food hardness to affect morphology during early growth as occurs among morphs of some fishes¹⁸. All heritabilities except for tarsus



were significantly different from zero ($P < 0.01$). Heritabilities ($\pm$ s.e.) and repeatabilities (%) for each character and generalized size and shape characters (principal component 1 and 2), are: wing length, 0.49 ± 0.17 , 29; lower mandible width, 1.13 ± 0.16 , 92; bill depth, 0.67 ± 0.14 , 88; upper mandible length, 1.25 ± 0.19 , 80; lower mandible length, 1.12 ± 0.27 , 82; PC1, 0.58 ± 0.10 ; PC2, 0.35 ± 0.11 . The number of other loci which may secondarily be involved in modifying the expression of the main locus is currently unclear.

12°15' E) to a breeding facility at the Riverbanks Zoological Park in Columbia, South Carolina. Pairs were housed under identical environmental conditions and fed a commercial finch diet¹⁰. Pedigrees (Fig. 2) show that all crosses produced offspring with either small or large bills, but not individuals with intermediate sized bills. Heterotypic pairs that produced mixed broods did so in a ratio not significantly different from 1:1 (21 small: 30 large, $\chi^2 = 0.64$, $P > 0.4$). Homotypic large pairs produced offspring in nearly a 3:1 ratio (16 l: 5 s), whereas all small homotypic pairs produced offspring with bill sizes like those of their parents. The distribution of lower mandible width in F_1 s produced from heterotypic pairs producing mixed broods is shown in Fig. 3. These results are consistent with the polymorphism being produced by a single autosomal di-allelic locus with complete dominance for large-bill.

Although *Pyrenestes* exhibits distinct bill morphs within single interbreeding populations with no apparent geographic segrega-

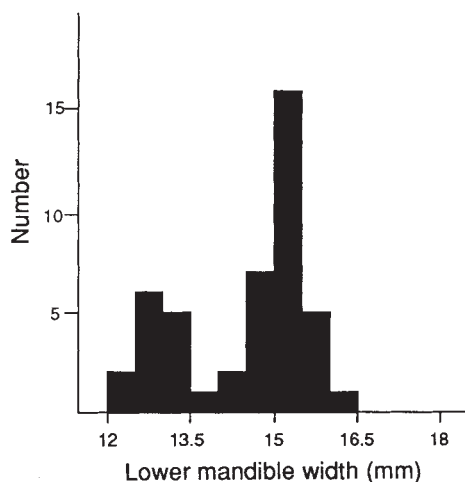


FIG. 3 Distribution of bill size in F_1 s produced from the heterotypic pairs producing mixed broods (Fig. 2). Lower mandible widths for the four pairs, sires and dams, respectively, are: 15.9 mm, 12.1 mm; 16.2, 12.4; 14.9, 12.7; 15.2, 12.6.

tion, an analysis of museum specimens revealed the occurrence of a third extremely large-billed form, occurring in ecotonal regions between forest and savanna⁵. In northern Cameroon, I found this megabilled form coexisting with small and large morphs (Fig. 4). The megabilled form fed primarily on a species of sedge (*S. racemosa*) with extremely hard seeds (mean hardness = 299 N, s.e. ± 11.9). A single breeding pair was found consisting of a small-billed male and a megabilled female, which is remarkable considering that megabilled forms are 31% heavier and have a bill over 47% larger¹¹.

In *Pyrenestes*, the precise role that disruptive selection plays in maintaining the polymorphism in an ecologically dynamic

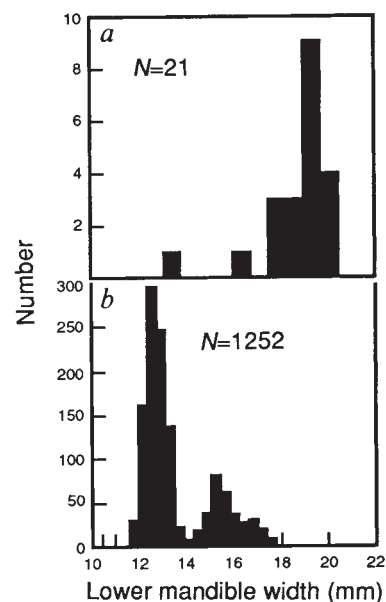


FIG. 4 a, Distribution of lower mandible width of adult males and females captured at Tibati (06°28' N, 12°38' E), in central Cameroon. Histogram shows the presence of small-, large- and megabilled individuals. b, The distribution of lower mandible width of adults of both sexes from the main study area in south central Cameroon is shown for comparison.

environment requires further research. But the apparent simple genetic basis of the polymorphism suggests that single mutations may have allowed populations to cross adaptive valleys^{12,13} in which larger morphs occupy separate adaptive peaks through differences in feeding performance on seeds of differing hardness. This hypothesis is supported by the most closely related species, *Spermophaga haematina*, exhibiting a bill similar in size and shape to the small morph of *P. ostrinus*, suggesting the ancestor of *Pyrenestes* also had a small bill⁶. Data further suggest that disruptive selection is important in producing and maintaining discrete polymorphisms² and that alternative adaptations may be maintained by polymodal selection¹⁴. Results also lend empirical evidence counter to the fisherian view that adaptations result primarily from allelic substitutions of slight effect at many loci^{15,16}, and supports recent views that major genes may be an important source of new adaptations¹². □

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1. Mayr, E. *Animal Species and Evolution* (Harvard Univ. Press, Cambridge, 1963).
2. Thoday, J. M. *Nature* **181**, 112 (1958).
3. Futuyma, D. J. *Evolutionary Biology* (Sinauer, Sunderland, MA, 1986).
4. Smith, T. B. *Nature* **329**, 717–719 (1987).
5. Smith, T. B. *Biol. J. Linn. Soc.* **41**, 381–414 (1990).
6. Smith, T. B. *Ecology* **71**, 1246–1257 (1990).
7. Smith, T. B. *Oikos* **60**, 76–82 (1991).
8. Smith, T. B. *Evolution* **44**, 832–842 (1990).
9. Schluter, D. *Evolution* **42**, 849–861 (1988).
10. Smith, T. B., Frampton, T. & Seibels, R. *Proc. 1990 Regional AAZPA* (1990).
11. Smith, T. B. *Proc. 8th Pan-African Ornithological Congr.* (Bujumbura, Burundi, in the press).
12. Orr, H. A. & Coyne, J. A. *Am. Nat.* **140**, 725–742 (1992).
13. Charlesworth, B. & Charlesworth, D. *J. theor. Biol.* **55**, 305–324 (1975).
14. West-Eberhard, M. J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 1388–1392 (1986).
15. Mayr, E. *The Growth of Biological Thought* (Harvard Univ. Press, Cambridge, 1982).
16. Fisher, R. A. *The Genetical Theory of Natural Selection* (Dover, New York, 1958).
17. Falconer, D. S. *Introduction to Quantitative Genetics* (Longman, London, 1981).
18. Meyer, A. *Oecologia* **80**, 431–436 (1989).

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Ecology of transgenic oilseed rape in natural habitats

M. J. Crawley, R. S. Hails*, M. Rees, D. Kohn & J. Buxton

Department of Biology, Imperial College, Silwood Park, Ascot, Berks SL5 7PY, UK

* Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, UK

CONCERNS about genetically engineered crop plants centre on three conjectural risks: that transgenic crop plants will become weeds of agriculture or invasive of natural habitats; that their engineered genes will be transferred by pollen to wild relatives whose hybrid offspring will then become more weedy or more invasive; or that the engineered plants will be a direct hazard to humans, domestic animals or beneficial wild organisms (toxic or allergenic, for example). Here we describe an experimental protocol for assessing the invasiveness of plants. The object is to determine whether genetic engineering for herbicide tolerance affects the likelihood of oilseed rape becoming invasive of natural habitats. By estimating the demographic parameters of transgenic and conventional oilseed rape growing in a variety of habitats and under a range of climatic conditions, we obtain a direct comparison of the ecological performance of three different genetic lines (control, kanamycin-tolerant transgenics and herbicide-tolerant transgenic lines). Despite substantial variation in seed survival,

plant growth and seed production between sites and across experimental treatments, there was no indication that genetic engineering for kanamycin tolerance or herbicide tolerance increased the invasive potential of oilseed rape. In those cases in which there were significant differences (such as seed survival on burial), transgenic lines were less invasive and less persistent than their conventional counterparts.

We used genetically engineered plants grown in natural habitats to compare the demography of transgenic and conventional lines of plants in a range of habitats throughout Great Britain in order to find out how ecological performance is affected by genetic engineering. To quantify the effect on the invasive potential, we estimated the finite rate of increase (λ) of different genetic lines under a variety of experimental conditions. Values of $\lambda > 1$ (the invasion criterion) predict that the plant will increase in abundance under the given set of environmental conditions; values of $\lambda < 1$ predict that the plants will decline to extinction^{1,2}. The model system was the crop plant *Brassica napus* subsp. *oleifera* (oilseed rape variety Westar), engineered to express tolerance of the antibiotic kanamycin and tolerance of the herbicide glufosinate (marketed as Basta in Europe and as Challenge in the UK). Kanamycin is used as a selectable marker in the transformation process and is not expected to confer any advantage in the field. Glufosinate herbicide works by interfering with the plant's ammonium metabolism and the expression of a glufosinate-tolerant phenotype allows oilseed rape to be sprayed with herbicide, killing weeds without harming the crop. As neither antibiotics nor herbicides were applied in the experiments, we predicted that the transgenic lines would not outperform the conventional plants. It was not clear, however, whether the expression of a transgenic phenotype would impose a measurable cost in terms of reduced ecological performance by the transgenic lines (the 'genetic baggage' hypothesis^{3,4}).

Oilseed rape is an annual or short-lived monocarpic perennial with a simple life cycle. Seeds germinate in autumn or in the following spring, flowering and seed ripening occur during summer and seed is shed in early autumn. The experiments involved spring sowing (Table 1), so the finite rate of increase can be written as

$$\lambda_1 = (1 - d_1 - g) + g(1 - d_2)\bar{F} \quad (1)$$

where d_1 is the proportion of seeds that die in one full year, g is the proportion of seeds germinating in the first spring, d_2 is the proportion of seeds that die over winter, and $\bar{F}$ is the mean number of seeds produced per seed that germinates^{2,5}. The first term on the right-hand side refers to the carry-over of seed from one year to the next, and the second term calculates the number of seeds produced by those plants that germinate and grow in competition with native vegetation. Separate experiments were carried out to evaluate each component: a seed burial experiment gave estimates of d_1 (and hence dormancy), whereas a seed sowing experiment provided estimates of germination, plant survival and fecundity. The experiments were carried out over three years in 12 different habitats at three sites within Great Britain (Sutherland, Cornwall and Berkshire); each experiment was replicated four times (details given in Table 1).

Seedling densities, adult plant densities and mean seed production per plant all varied significantly between years and between habitats within years (Table 1). There was no significant effect of genetic line on any of the demographic parameters, nor were there any interactions between sites, years and genetic line. Some of our experimental treatments had major effects on plant demography (cultivation to reduce interspecific competition, fencing to exclude vertebrate herbivores, chemical exclusion of molluscs), whereas others had none (exclusion of insect herbivores or fungal pathogens); these results will be presented elsewhere.

Seeds buried in each of the habitats at two depths (2 cm and 15 cm) were retrieved after 12 and 24 months and their fate

TABLE 1 Population densities of seedlings and mature plants and fruit production of three lines of oilseed rape in 12 habitats in three sites

	Habitat number	1990			1991			1992		
		Control	Kanamycin	Basta	Control	Kanamycin	Basta	Control	Kanamycin	Basta
Seedlings	1	153	142	148	7	M	1	129	113	127
	2	104	95	110	51	M	29	87	148	104
	3	61	72	48	70	M	39	29	19	28
	4	62	56	19	40	M	29	285	386	337
	5	11	4	2	85	90	67	34	37	34
	6	0	2	1	158	150	104	79	78	81
	7	15	17	20	61	43	32	65	73	94
	8	106	122	104	6	2	4	302	247	248
	9	50	44	18	32	17	14	78	110	72
	10	43	36	39	41	19	32	147	123	128
	11	204	145	219	67	84	100	142	167	129
	12	120	116	147	67	70	44	143	117	152
Adults	1	78	47	52	3	M	1	43	44	45
	2	8	0	6	0	M	0	0	0	0
	3	1	0	0	8	M	5	0	0	0
	4	0	0	0	1	M	5	3	0	0
	5	0	0	0	1	1	0	0	0	0
	6	0	0	0	11	2	1	0	0	0
	7	15	3	13	2	0	0	12	24	4
	8	12	14	7	0	0	0	107	108	90
	9	0	0	0	14	15	2	3	19	5
	10	0	0	0	18	7	5	3	5	4
	11	51	27	67	30	30	35	13	29	16
	12	16	28	36	32	43	29	13	13	11
Fecundity	1	2,214	848	1,380	0	M	0	1,280	919	1,064
	2	9	0	14	0	M	0	0	0	0
	3	2	0	0	0	M	0	0	0	0
	4	0	0	0	0	M	0	1	0	0
	5	0	0	0	0	0	0	0	0	0
	6	0	0	0	0	0	0	0	0	0
	7	90	27	267	0	0	0	44	53	14
	8	86	145	39	0	0	0	2,250	1,882	1,341
	9	0	0	0	1	1	0	3	30	8
	10	0	0	0	0	0	0	2	5	5
	11	2,349	2,386	1,499	162	8	158	245	912	393
	12	82	107	90	61	80	31	90	54	21

2,320 seeds were sown in the spring of each year (1,856 seeds sown per habitat in 1990). Fecundity is the total number of siliques produced (each silique contains between 0 and 32 seeds; mean healthy seeds per silique, 6.54). The geographic locations were Cornwall in southwest England (mild winters, early start to the growing season); Berkshire in southeast England (a more continental climate with cold winters and dry summers); Sutherland in northeast Scotland (a late start to the growing season, but long summer days). Four habitats were chosen at each location to represent a characteristic set of ecological conditions (sun and shade, wet and dry substrates). The same experiment was carried out at each of the 12 habitats. Four replicate plots measuring 25 m × 25 m were selected at random within each habitat. Half the plot was fenced to exclude rabbits and larger vertebrate herbivores. Half the area was cleared of all native vegetation and cultivated to a depth of 15 cm by hand-digging (the reduced competition treatment), giving 4 treatment combinations (fenced-cultivated, fenced-uncultivated, unfenced-cultivated and unfenced-uncultivated). The outer perimeter of the cultivated plots was trenched to a depth of 25 cm to sever all roots of surrounding vegetation. Within each of the 12.5 × 12.5 m subplots, 3 strips measuring 15 m × 50 cm were laid out in each of 3 years (one for each of the three genetic lines). Within each strip, seeds were sown at two densities (high density corresponds to 10 seeds in 1 cm² in the centre of a 25 × 25 cm quadrat; low density corresponds to 9 regularly spaced seeds in 625 cm²). Chemical pesticides were applied in a factorial design (plus and minus insecticide (heptenophos 3.3% w/w and permethrin 0.8% w/w foliar-applied, plus pirimiphos-methyl 80 g/l soil-applied), plus and minus fungicide (propiconazole 5 g/l⁻¹), and plus and minus molluscicide (metaldehyde pellets) in 1991 and 1992. In 1990, the glufosinate-tolerant line was 65% homozygous transgenic, 27% heterozygous transgenic and 8% non-transgenic; in 1991 and 1992 the seeds were all inbred transgenic; (data confirmed by Southern blot analysis^{8,9}; PGS Belgium). Differences between seedling densities were significant across habitats ($F_{11,108}=15.8$ (in 1990), 12.2 (1991) and 17.9 (1992); $p < 0.001$; Glim with Poisson errors corrected for overdispersion), but not between genetic lines. Differences between adult plant densities were assessed by computing the proportion of seedlings that survived to flowering; mortality varied significantly with habitat but not with transgenic line ($F_{11,108}=3.78$ (in 1990), 6.72 (1991) and 10.13 (1992); $p < 0.001$; Glim with binomial errors). Fecundity (total siliques per quadrat) varied significantly between habitats but not with transgenic line ($F_{11,108}=28.8$ (in 1990), 6.39 (1991) and 49.1 (1992); $p < 0.001$; Glim with normal errors on log-transformed (pod numbers + 1)). M, missing data (shortage of kanamycin seed in Cornwall in 1991); Habitat codes and site details in R.S.H. *et al.*, manuscript in preparation.

determined (Table 2 legend). The weedy relative *Sinapis arvensis* was included as a barometer of local seed-preservation conditions; this species is capable of protracted dormancy^{5,6}, and low rates of charlock viability would show whether certain soils were inimical to brassica seed survival. Charlock showed high survival in all habitats (mean survival after the first year of 60%, compared with mean survival of 2% for non-transgenic oilseed rape), and the two transgenic lines of oilseed rape had significantly lower survival than non-transgenics (Table 2). But it is not possible to attribute the reduction in seed survival unequivocally

to genetic engineering, because maternal effects and other factors involved in the production of the different plant lines might have influenced the properties of the seeds.

The parameters estimated from the experiments were combined to obtain an estimate of the finite rate of increase, λ , for each genetic line under each of the treatments in each of the 12 habitats. As predicted⁷, interspecific plant competition proved to be the main determinant of λ ; Fig. 1 shows estimates obtained in each of three years from plots that were cleared of all vegetation and cultivated before seed sowing, compared with neigh-

bouring uncultivated plots supporting intact native vegetation. There were no significant differences in the finite rate of increase between the genetic lines on cultivated plots for any sites in any year. In two of the three years, all three genetic lines had $\lambda > 1$ on the cultivated plots, predicting that rape populations would increase (maximum λ values for the three genetic lines were 19.1, 15.7 and 11.5 for the control, kanamycin- and glufosinate-tolerant lines in 1990). These λ values are based on the seeds starting life in a cultivated, competition-free environment. In contrast, the seeds produced by the first generation of our experimental plants were shed into an environment in which the competing perennial vegetation had had a full growing season to recover from cultivation. If we compute a new estimate of λ based on the number of self-sown seedlings in year $t+1$ to the number of seedlings arising from the experimental sowing, the picture is somewhat different:

$$\lambda_2 = \frac{\text{seedlings of generation 2}}{\text{seedlings of generation 1}} \quad (2)$$

By this yardstick, the invasion criterion was not realized in any of the habitats (Fig. 1). There was a positive correlation between rape seed production and the rate at which the native vegetation recolonized the cultivated plots (Spearman's rank correlation = 0.74; $n = 48$, $p < 0.05$); the more fertile the plots, the higher the rape seed production but the more quickly the open ground was colonized by native perennials, thereby limiting the opportunities for rape recruitment. There was some recruitment of seedlings in 1991 from seeds produced in 1990, but none in 1992

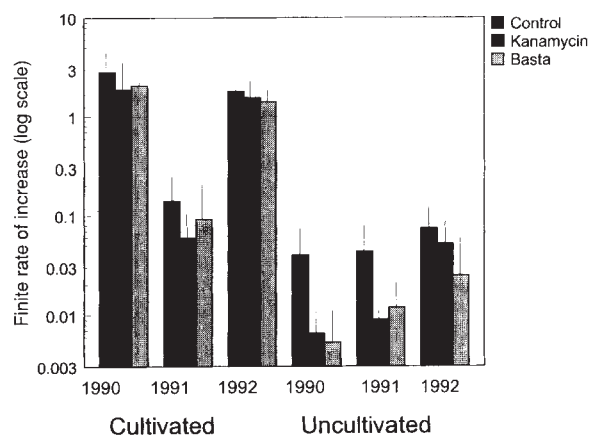


FIG. 1 The finite rate of increase for 3 genetic lines of oilseed rape in three separate experiments (1990, 1991 and 1992) in cultivated and uncultivated plots averaged over 12 habitats. Populations for which $\lambda_1 > 1$ would be expected to increase in abundance. Values of $\lambda_1 > 1$ were observed on cultivated plots in 1990 and 1992, but there was no significant difference between the transgenic and conventional lines in any years or any experimental treatments on the cultivated plots (bootstrapped 95% confidence intervals estimated over 3,000 randomizations). Rape seed sown into undisturbed vegetation never produced values of $\lambda_1 > 1$, despite the fact that a few individual plants grew vigorously and set substantial seed under apparently inhospitable conditions. Significant differences in λ_1 on the uncultivated plots in 1990 and 1991 reflect differences between the genetic lines in the probability of seed carry-over (Table 2). These differences cannot be attributed unequivocally to the transgenic constructs because seeds differed in other ways, notably in the cultural treatment of the maternal plants. A second estimate (λ_2) is based on the ratio of seedlings in successive generations and shows $\lambda_2 < 1$ for all genetic lines in all habitats ($0.001 < \lambda_2 < 0.007$ for 1990–1991 (maximum $\lambda_2 = 0.045$ for the control line on cultivated plots); all values for 1991–1992 $\lambda_2 = 0$). The large difference between the two estimates of λ results from the rapid regrowth of perennial plants on the cultivated plots which reduces the opportunity for subsequent seedling recruitment. Thus, the difference between persistence and extinction depends on the externally determined rate of disturbance as well as on the survival and fecundity of the rape plants.

from seed produced from the poor year of 1991 (Fig. 1). Accordingly, we predict that introduced oilseed rape is heading for local extinction in all experimental treatments in every habitat. This result highlights the importance of competition-free gaps for successful recruitment of rape from seed in natural habitats. It also suggests that those habitats (like motorway verges) where oilseed rape appears to be a permanent feature of the spring flora, are subject to regular disturbance (for example, from grass-cutting machinery) and/or that they experience an annual input of seed spilled in transit.

In summary, there is no evidence that rape is invasive of undisturbed natural habitats, and no evidence that transgenic lines of rape are more invasive of, or more persistent in, disturbed habitats than their conventional counterparts. Where there were significant differences between the genetic lines, the transgenics performed less well than the non-transgenics, but overall our data provide little support for the 'genetic baggage' hypothesis³; there were no substantial costs involved in expressing a kanamycin- or glufosinate-tolerant phenotype. Although these results

TABLE 2 Seed burial and seed dormancy for conventional and transgenic oilseed rape and a weedy relative (charlock, *Sinapis arvensis*)

	Habitat number	Control	Kanamycin	Basta	Charlock
Year 1	1	60	1	1	302
	2	3	2	0	144
	3	5	2	0	243
	4	0	0	4	180
	5	37	4	10	218
	6	16	11	0	322
	7	0	0	1	239
	8	1	0	1	256
	9	0	0	1	50
	10	28	2	1	263
	11	16	6	0	324
	12	6	0	2	341
Year 2	1	6	0	0	267
	2	0	1	0	29
	3	0	0	0	79
	4	0	0	0	17
	5	2	0	0	134
	6	1	0	0	71
	7	1	0	0	232
	8	0	0	0	141
	9	9	1	0	2
	10	0	0	0	12
	11	5	2	0	229
	12	0	1	0	352

Table shows the total number of viable seeds (out of 400) alive after burial for 12 and 24 months, averaged over 2 depths for 3 genetic lines of oilseed rape (control, kanamycin-tolerant and herbicide-tolerant) and for charlock. Flat nylon-mesh bags (1-mm mesh size) containing 50 seeds were buried in April (May in Sutherland) 1990 at 2 cm or 15 cm below the surface of uncultivated native vegetation inside each of the 4 fenced plots (12.5 × 12.5 m) in each of 4 habitats at 3 sites (Table 1). The bags were recovered in April (May in Sutherland) 1991 and 1992, and the seeds were dissected and sorted into three categories: germinated, viable and dead. For oilseed rape, seed survival over the first year was less than 2% in most cases, but was significantly greater for controls than transgenics ($F = 29.36$, d.f. = 2,48; $P < 0.01$, Glim with binomial errors). The two transgenic lines were not significantly different from one another in their dormancy rates. Seed survival of the weedy relative was much higher than the crop plant in all habitats (average 60.7%), and was significantly increased with deeper burial ($\chi^2 = 13.7$, d.f. = 1; $P < 0.05$, Glim with binomial errors). There was no significant effect of depth of burial on rape seed survival. Survival of control seed during the second year (average 14%) was somewhat higher than during the first, and represented 0.5% of the original buried seed. Only 5 viable seeds were recovered from the two transgenic lines after burial for 2 years (estimated second year survival, 10%; overall 2-year survival, 0.1%). Differences in seed survival after burial are the cause of the significant differences in λ_1 between genetic lines on the uncultivated plots in 1990 and 1991 (Fig. 1).

suggest that herbicide-tolerant oilseed rape poses no greater threat to the environment than the conventional crop, it would be prudent to reserve judgment on the risks that might be posed by other crop species or different transgenic constructs (such as transformations like drought tolerance or pest resistance, which might be expected to enhance plant performance in natural habitats). □

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1. Crawley, M. J. *Phil. Trans. R. Soc. Lond. B* **314**, 711–731 (1986).
2. Rees, M., Kohn, D., Halls, R., Crawley, M. & Malcolm, S. in *Biological Monitoring of Genetically Engineered Plants and Microbes* (ed. MacKenzie, D. R. & Henry, S. C.) 9–24 (USDA and Clemson Univ., South Carolina, 1991).
3. Regal, P. J. *Trends Ecol. Evol.* **6**, S47–S49 (1988).
4. National Academy of Sciences *Introduction of Recombinant DNA-engineered Organisms into the Environment* (National Academy, Washington, 1987).
5. Rees, M. & Long, M. J. *Am. Nat.* **139**, 484–508 (1992).
6. Rees, M. & Long, M. J. *Am. Nat.* **141**, 233–262 (1993).
7. Crawley, M. J. *Phil. Trans. R. Soc. Lond. B* **330**, 123–140 (1990).
8. Thompson, C. J. *et al.* *EMBO J.* **6**, 2519–2523 (1987).
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbour Press, New York, 1989).

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Spatial working memory in humans as revealed by PET

John Jonides*, Edward E. Smith*, Robert A. Koeppel†, Edward Awh*, Satoshi Minoshima† & Mark A. Mintun‡

Departments of * Psychology and † Internal Medicine, University of Michigan, Ann Arbor, Michigan 48109, USA
‡ Division of Nuclear Medicine, Presbyterian University Hospital, DeSoto at O'Hara Streets, Pittsburgh, Pennsylvania 15213, USA

THE concept of working memory is central to theories of human cognition because working memory is essential to such human skills as language comprehension and deductive reasoning^{1–4}. Working memory is thought to be composed of two parts, a set of buffers that temporarily store information in either a phonological or visuospatial form, and a central executive responsible for various computations such as mental arithmetic^{5,6}. Although most data on working memory come from behavioural studies of normal and brain-injured humans⁷, there is evidence about its physiological basis from invasive studies of monkeys^{8–10}. Here we report positron emission tomography (PET) studies of regional cerebral blood flow in normal humans that reveal activation in right-hemisphere prefrontal, occipital, parietal and premotor cortices accompanying spatial working memory processes. These results begin to uncover the circuitry of a working memory system in humans.

The two main conditions of the experiment are illustrated in Fig. 1. The volunteers were trained on the perception and memory tasks and then prepared for PET scanning. The experiment consisted of six scans: two perception conditions, two memory conditions, and two scans of other conditions not relevant to the present experiment. Figure 2 illustrates the protocol for a scan.

Subjects were quite accurate in their behavioural performance in this task, making no errors in the perception condition, and only 15.8% errors in the memory condition. Of these errors, roughly half occurred on those trials in which the probe did not encircle a target location. Interestingly, these 'miss' errors were more frequent the closer the probe was to a previously occupied

location (28.9% of them occurred for 'near' probes versus 7.5% for 'far' probes). This is to be expected if subjects used a spatial memory buffer to mediate their performance, making a probe that appeared relatively close to the location of a previous target difficult to reject on the basis of having encircled that location.

The major difference between the perception and memory conditions was that only the latter required subjects to store location information; hence, the differences between these two conditions provide information about the localization of processes associated with memory. Figure 3 presents four brain images showing the brain activation levels that resulted from subtraction of the perception from the memory condition.

The top left image in Fig. 3 shows one of the areas of reliable activation, in the right prefrontal cortex. This focus is consistent with reports of single-cell activity in monkeys in a similar area, in the vicinity of the principal sulcus¹⁰. But the reported activity in monkeys is bilateral, in contrast to the largely unilateral activation in our human subjects. A second area of activation shown in the bottom left image of Fig. 3 was centred in the posterior portion of the parietal cortex of the right hemisphere.

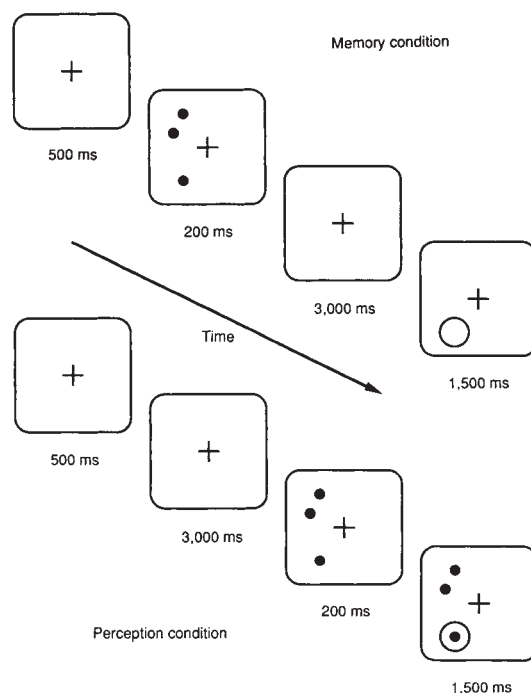
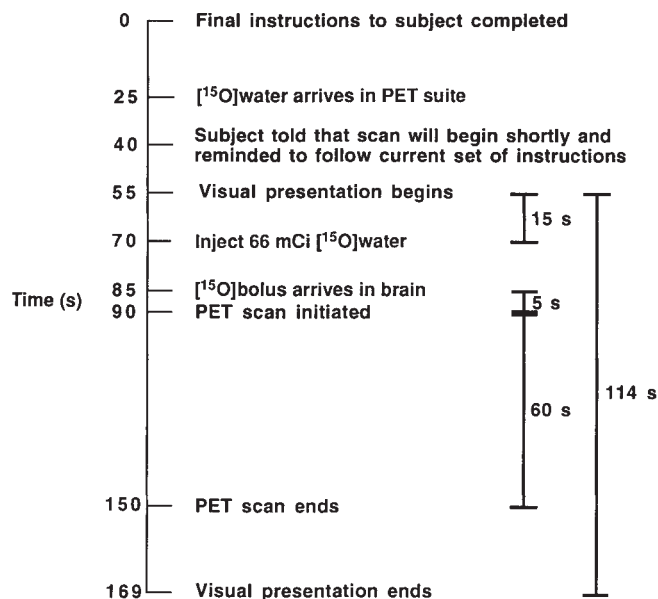


FIG. 1 The upper part of the figure illustrates the events in the 'memory' condition of the experiment. Subjects began by fixating a cross in the centre of the screen for 500 ms. The cross was then supplemented by three dots appearing on the circumference of an imaginary circle of 14 degrees diameter, centred on the cross. The dots remained in view for 200 ms (too short an interval to permit a successful saccade to the dots on average), following which the fixation cross continued to appear for a retention interval of 3,000 ms. This was followed by a probe for location-memory which consisted of a single outline circle that either encircled the location of one of the previous dots (with probability 0.50) or not. The probe circle was presented for 1,500 ms; during this interval, subjects pressed a response button once or twice to indicate whether or not the probe marked the location of a dot. The probe circle was either centred directly over the previous location of a dot, or it missed the nearest dot location by 15–40 degrees. The lower part of the figure shows the events on each trial of the 'perception' condition. Trials again began with a fixation cross, which remained in view for 3,500 ms (the duration of the fixation plus retention intervals in the memory condition). This was followed by three dots presented for 200 ms, followed immediately by an interval of 1,500 ms during which the three dots and probe circle were presented simultaneously. As in the memory condition, subjects pressed a response button once or twice to indicate whether or not the probe encircled a dot. The order of administration of conditions was counterbalanced across subjects.

Protocol for a PET scan



This area has previously been reported to be engaged by visual tasks that require object localization^{11,12}. A third area of activation (shown in the top right image of Fig. 3) is in the right occipital cortex. This area has been implicated in the creation of images in humans^{13,14}. The final focus of activation, illustrated in the bottom right image, is in right premotor cortex. We do not believe that activation in this region is due to the motor requirement of the present task (responding with button pushes) because this requirement was identical in both the perception and memory conditions and therefore should have been subtracted out. More importantly, the premotor activation revealed in our data is in the right hemisphere, yet all subjects responded with their right hands.

The statistically significant areas of increased activation reported above are all located in the right hemisphere. There was increased activation as well in the homologous areas in the left hemisphere (as suggested by examination of some of the images of Fig. 3), although none of the areas in the left hemisphere reached statistical significance by our criterion. Nevertheless, there is reason to be cautious about the localization of the relevant processes to only one hemisphere (see ref. 15 for related

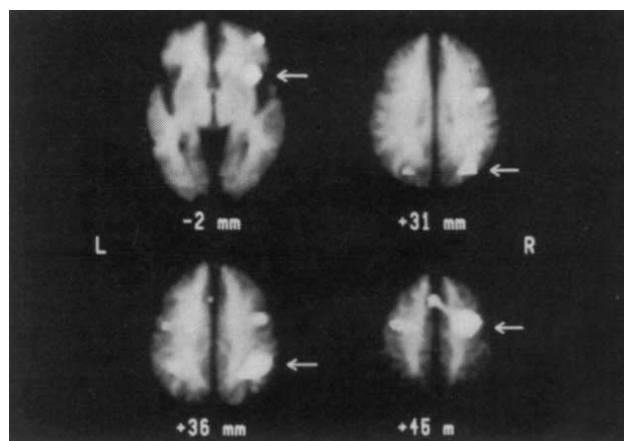
FIG. 2 Each scan consisted of 20 trials, with the first 3 presented before the injection of the radionuclide (the total duration of these 3 trials was ~15 s). Immediately after these trials, an intravenous bolus administration of 66 mCi of [¹⁵O]water was given, after which ~15 s elapsed before the radionuclide reached the brain. Trials continued to be administered during this interval. Recording of activity began 5 s after the count rate was observed to increase above the background level and continued for 60 s thereafter. Injections for subsequent scans were separated by 14-minute intervals (seven half-lives), permitting the ¹⁵O to decay to less than a 1% background level.

evidence).

The patterns of brain activation revealed in this experiment suggest possible models of spatial working memory processes that are broadly consistent with the modal conceptualization of working memory derived from behavioural experiments⁵. One possible model postulates that subjects create an internal image of the target dots at the time these targets are presented, using mechanisms in the occipital lobe. An internal image of the dots might correspond to a matrix-like representation that includes information about target location for all the dots simultaneously. This image might then be used to calculate the dot coordinates by mechanisms in parietal cortex. Storage processes of the prefrontal cortex might then be responsible for keeping the image in memory during the retention interval. Further tests of this and similar models will be possible with further refinements of the tasks given to subjects while their brain activity is recorded.

The present experiment provides data once again implicating lateral prefrontal cortex in the maintenance of spatial information for short periods of time, thereby extending previous research conducted on monkeys¹⁰. Although it is becoming increasingly clear that prefrontal cortex is involved in working

FIG. 3 PET images of the four statistically significant activation sites, each superimposed on a magnetic resonance image of a composite brain for the purpose of illustrating the anatomical localization of activation foci. The images represent the subtraction of the perception from the memory condition across 18 subjects. The areas of activation highlighted by arrows are those that exceeded a level of statistical significance of $P < 0.05$ by t -test after correction for multiple comparisons. All four significant foci of activation were in the right hemisphere. These were in prefrontal cortex (Brodmann's area 47, stereotaxic coordinates: -35, 19, -2), parietal cortex (Brodmann's area 40, stereotaxic coordinates: -42, -40, 36), occipital cortex (Brodmann's area 19, stereotaxic coordinates: -30, -76, 31), and premotor cortex (Brodmann's area 6, stereotaxic coordinates: -34, -1, 45) (coordinates are presented in the order: left to right, posterior to anterior, and ventral to dorsal). The data were derived by the following method. The PET images for each subject were transformed to a stereotaxic coordinate system^{17,18} and standardized to an atlas brain by linear scaling¹⁹. After normalizing pixel values for global flow rate differences among scans²⁰, the data were averaged across the 18 subjects, giving mean and variance values for each condition. The average image for the perception condition was subtracted from that of the memory condition to reveal differences in activation between these conditions. The difference image was analysed for statistical significance on a pixel-by-pixel basis using t -statistics, followed by a multiple-comparison adjustment based on the Bonferroni method²¹. A one-tailed adjusted value of $P < 0.05$ was used as a criterion for reliability.



memory, it is not yet clear what its role is. One obvious possibility (assumed in the preceding model) is that the neuronal activity recorded in prefrontal cortex is itself the internal representation of spatial location that is maintained during a retention interval. According to this view, spatial location may be coded by individual neurons, or it may be coded by populations of such neurons, as is apparently characteristic of other cortical areas¹⁶. It is also possible that the prefrontal activity that we and others have recorded represents a pointer or index to other circuitry that is responsible for maintaining the actual engram for spatial location. This other circuitry may itself be coherent and well localized (for example, consisting of an image stored in occipital cortex), or it may be distributed among a number of interlinked areas of cortex. □

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1. Daneman, M. & Carpenter, P. A. *J. exp. Psychol. Learn. Mem. Cogn.* **9**, 561-584 (1983).
2. Baddeley, A. D., Papagno, C. & Vallar, G. *J. Mem. Lang.* **27**, 586-595 (1988).
3. Carpenter, P. A., Just, M. A. & Shell, P. *Psychol. Rev.* **97**, 404-431 (1990).
4. Holding, D. H. *The Psychology of Chess Skill* (Erlbaum, Hillsdale, NJ, 1985).
5. Baddeley, A. D. *Science* **255**, 556-559 (1992).
6. Baddeley, A. D. *Working Memory* (Oxford Univ. Press, Oxford, 1986).
7. Vallar, G. & Shallice, T. (eds) *Neuropsychological Impairments of Short-Term Memory* (Cambridge Univ. Press, Cambridge, 1990).
8. Fuster, J. M. *Hum. Neurobiol.* **4**, 169-179 (1985).
9. Passingham, R. E. *Behav. Neurosci.* **99**, 3-21 (1985).
10. Goldman-Rakic, P. S. In *Handbook of Physiology: The Nervous System* (ed. Plum, F.) (Am. Physiol. Soc., Bethesda, MD, 1987).
11. Haxby, J. V. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 1621-1625 (1991).
12. Chaffee, M., Funahashi, S. & Goldman-Rakic, P. S. *Soc. Neurosci. Abst.* **15**, 786 (1989).
13. Goldenberg, G., Podreka, I., Steiner, M. & Willmes, K. *Neuropsychology* **25**, 473-485 (1987).
14. Kosslyn, S. M. *et al. J. cogn. Neurosci.* (in the press).
15. Farah, M. J. *Psychol. Rev.* **95**, 307-317 (1988).
16. Georgopoulos, A. P., Schwartz, A. B. & Kettner, R. E. *Science* **233**, 1416-1419 (1986).
17. Minoshima, S., Berger, K. L., Kee, K. S. & Mintun, M. A. *J. nucl. Med.* **33**, 1579-1585 (1992).
18. Minoshima, S. *et al. J. nucl. Med.* **34**, 322-329 (1993).
19. Talairach, J. & Tournoux, P. *A Co-planar Stereotaxic Atlas of a Human Brain* (Thieme, Stuttgart, New York, 1988).
20. Fox, P. T., Fox, J. M., Raichle, M. E. & Burde, R. M. *J. Neurophysiol.* **54**, 348-369 (1985).
21. Friston, K. J., Frith, C. D., Liddle, P. F. & Frackowiak, R. S. J. *J. Cereb. Blood Flow Metab.* **11**, 690-699 (1991).

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Extent to which homology can constrain coding exon junctional diversity in *V(D)J* recombination

Rachel M. Gerstein & Michael R. Lieber*

Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305-5324, USA

AMONG site-directed DNA recombination systems, *V(D)J* recombination is noteworthy in that identical reactants yield different recombination products at the junction of joined segments. This variation is the basis for diversity at the base of antigen receptor binding pockets and corresponds to *V(D)-J* DNA junctions. An abundance of certain junctions has been noted¹⁻⁵. It has been proposed that these junctions are favoured because they occur where short regions of homology in participating coding ends might align preferentially¹. Here we use a system that is entirely free from cellular selection to show that the diversity of coding joints can be severely restricted when the coding ends participating in the reaction have short regions of homology. This constraint on diversity is diminished but not eliminated by terminal deoxynucleotidyl transferase, a mechanistic feature that has implications for the establishment of the immune repertoire.

We have assessed the frequency of coding end homology

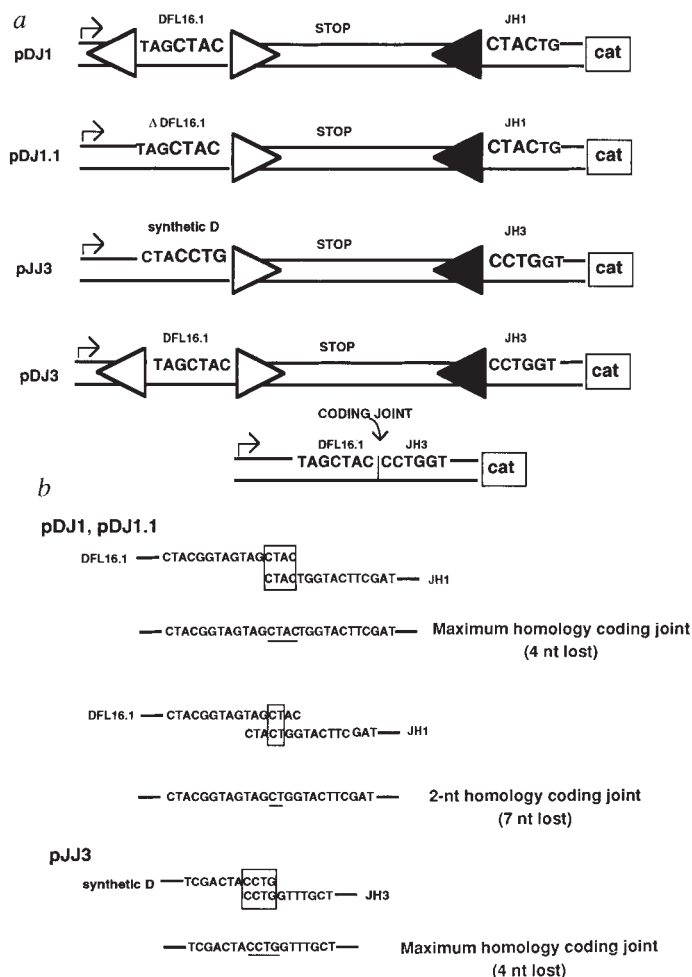


FIG. 1 *DJ* recombination substrates and their recombinant products. *a*, Substrates. Essential features of these recombination substrates have been described¹³. *V(D)J* recombination signal sequences (the signal with the 12-bp spacer is depicted as an open triangle and the 23-bp spacer signal is shown as a filled triangle) are separated by a stop sequence, the prokaryotic transcription terminator *oop*, and are placed between the chloramphenicol acetyltransferase gene (*cat*) and a prokaryotic promoter (*P_{lac}*), represented by an arrow. A small portion of the coding end nucleotide sequence is displayed. After excision of the signals and *oop* during *V(D)J* recombination in mammalian cells, the recombinant plasmid expresses *cat* upon transformation of *Escherichia coli*. Substrates contain the β -lactamase gene, which confers ampicillin resistance in *E. coli*. Therefore, recombinants can be selected on ampicillin/chloramphenicol plates. *b*, Illustration of coding joints. The maximum homology coding joint is formed when the 4-nucleotide (nt) region of sequence homology at the coding ends of pDJ1, pDJ1.1 or pJJ3 (boxed) is used in coding joint formation. The 2-nucleotide homology (boxed) coding joint shown results when 2, 3 or 4 nucleotides are lost from one coding end and 5, 4 or 3 are lost from the other coding end of pDJ1, pDJ1.1. Retained homology is underlined.

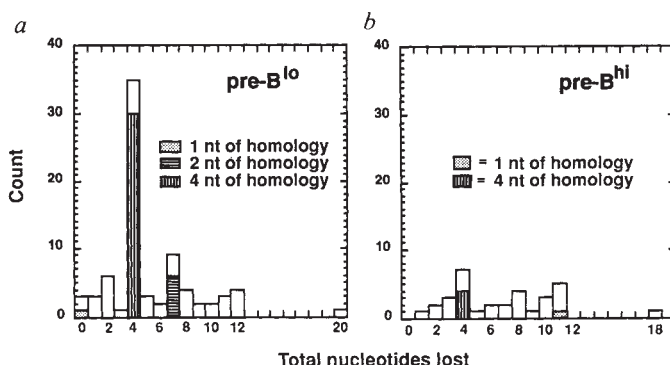
METHODS. pDJ1 was constructed by cloning oligonucleotides containing the DFL16.1 and JH1 of the BALB/c IgH locus into the *SalI* and *BamHI* sites, respectively, of pJH298 (ref. 13). pDJ1.1 is essentially the same as pDJ1, except that the Δ DFL16.1 element does not contain a signal sequence 5' of the coding sequence and does contain consensus *V(D)J* recombination signal sequences¹². This modification was necessary to obtain coding joints in fibroblasts co-transfected with RAG1 and RAG2 expression vectors. pDJ3 contains the same D element as pDJ1 and has an oligonucleotide containing JH3 from the BALB/c IgH locus inserted in the *BamHI* site. Construction of substrates containing DFL16.1, JH1, and JH3 are detailed in ref. 27. pJJ3 is a modification of pDJ3 in which the *SalI* insert has been replaced with an oligonucleotide (5'-TCGACTACCTGCACAGTGTCTATCCATCAGCAAAA-CCTGCAG-3') that creates a synthetic D element.

* To whom correspondence should be addressed.

FIG. 2 The distribution of coding joints formed by pDJ1 in pre-B-cell lines. *a*, pDJ1 recombinants isolated from pre-B^{lo}. Sequence data are represented as total number of nucleotides lost from the coding ends of pDJ1 during V(D)J recombination in transiently transfected pre-B^{lo} cells. Seventy-seven coding joints are shown (23 junctions have N or P nucleotide insertions). Each junction was isolated from independent transfections. Most coding joints that have lost a total of 4 nucleotides represent the maximum homology junction (filled with vertical bars). *b*, pDJ1 recombinants isolated from pre-B^{hi}. Thirty-two coding joints were compiled as for *a*. All are independent events isolated from 8 separate transfections; 19 are NP⁺. Of the 7 cases of 4-nucleotide loss, 4 recombinants are NP⁺ and represent the maximum homology junction (filled with vertical bars). The distributions of data in *a* and *b* were compared using the Kolmogorov-Smirnov test ($P=0.158$). This unexpectedly high P value may be due to the small sample size in *b*. The data from *a* were significantly different from those in Fig. 4c, ($P=0.006$), and from the combined data of Fig. 4a and *b* ($P=0.004$). Therefore the high frequency of the maximum homology coding joint in pre-B^{lo} does skew the distribution of coding joints in a statistically significant manner.

METHODS. pre-B^{lo}, also known as 22D6, is an Abelson murine leukaemia virus-transformed pre-B line derived from mouse fetal liver²⁸; pre-B^{hi} is also known as 1-8 (ref. 28), a pre-B line transformed with the same retrovirus and derived from adult mouse bone marrow. Transfection and subsequent selection of recombinants was as described in refs 13 and 27. The coding joints of recombinant plasmids were sequenced using a 20-nucleotide primer complementary to the 5' end of *cat*, using Sequenase according to the manufacturer's (USB) protocol.

usage in V(D)J recombination reactions by analysing recombinants generated on extrachromosomal substrates transfected into murine pre-B-cell lines. The recombination substrates used retain DJ coding joints after undergoing V(D)J recombination (Fig. 1). Substrate pDJ1 contains DFL16.1 and JH1 from the immunoglobulin heavy-chain locus. The 3' end of the DFL16.1 coding sequence and the 5' end of the JH1 coding sequence share four nucleotides. Substrates were transiently transfected into pre-B cells, collected, and independent recombinants isolated (Fig. 1). Among coding joints isolated from pre-B^{lo}, a fetal liver-derived pre-B-cell line expressing low levels of terminal deoxynucleotidyl transferase (Tdt)⁶, a particular coding joint is found in 30 of 54 (55%) reaction products that lack Tdt-dependent N-nucleotide regions (ref. 6 and refs therein; ref. 7) or P-nucleotide insertions (template-dependent inverted repeats⁸) (Fig. 2). An absence of both types of insertion is designated as NP⁻. This joint, termed the maximum homology coding joint, retains one of the 4-nucleotide homology blocks (and is therefore designated as a -4 coding joint), which is the expected product if the maximum homology is used to align the coding ends. These data represent the first assessment of the extent to which



sequence homology can clearly determine reaction outcome.

Coding joint products of pDJ1 were also isolated from pre-B^{hi}, a cell line expressing higher levels of Tdt than pre-B^{lo}. The frequency of the maximum homology coding joint is lower, although this junction did occur at a frequency of 4 of 32 (12%) (Fig. 2). The frequency of coding joints from pre-B^{hi} with a total nucleotide loss of four is highest (30%) in the NP⁻ subgroup of recombinants. These data indicate that the level of Tdt activity affects how often homology is apparent in the outcome at V(D)J coding joints.

To examine further the extent of homology utilization in the presence or complete absence of Tdt, we co-transfected a hamster fibroblast line with pDJ1.1 (Fig. 1), RAG1 and RAG2 expression vectors^{9,10} and, with or without pTdt, a Tdt expression vector⁷. As fibroblasts activated for V(D)J recombination form coding joints inefficiently¹¹, we designed pDJ1.1 to be more efficient for coding joint production by providing it with consensus signals¹² and omitting the 5' recombination signal sequence. A 33% (16 of 48 NP⁻ joints) incidence of maximum homology coding joints was observed in the absence of Tdt (Fig. 3). Two of 39 coding joints isolated from fibroblasts

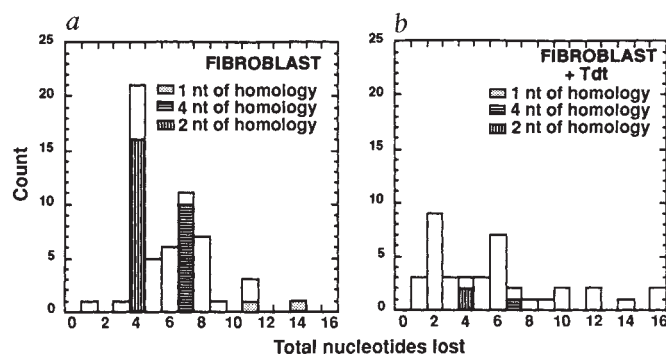
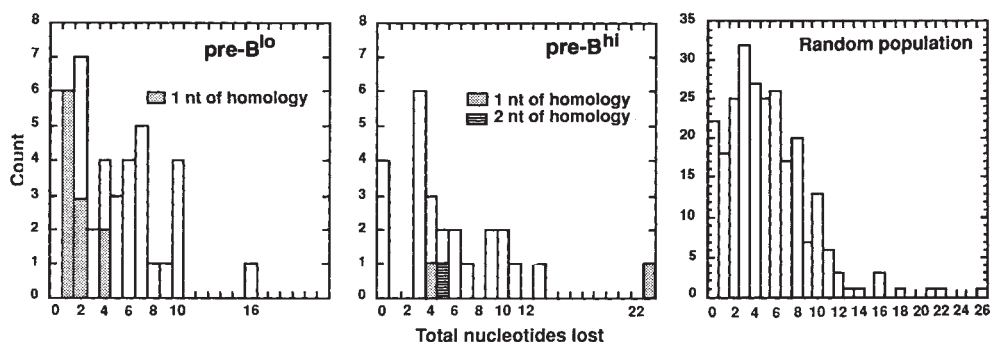


FIG. 3 The distribution of coding joints formed by pDJ1.1 in RAG1 and RAG2 co-transfected fibroblasts. *a*, pDJ1.1 recombinants isolated from hamster fibroblast line 4364A. Results are a compilation of 57 coding joint sequences, each isolated from separate transfections. Nine junctions have insertions: 8 P-nucleotide insertions and 1 with an insertion of a single A. *b*, pDJ1.1 recombinants isolated from hamster fibroblast line 4364A, co-transfected with pTdt. Thirty-nine sequences were compiled from recombinants isolated from two transfections. Nine junctions are without insertions. Two of these are the maximum homology joint. Data in *a* and *b* were compared using the Kolmogorov-Smirnov test ($P=0.002$); those in *a* were also compared with results shown in Fig. 4c ($P<0.0001$), and are also not significantly different from the data from Fig. 2a ($P=0.113$).

METHODS. 4364A, a proline and glycine auxotroph of CHO (ref. 29), was maintained in RPMI 1640 with 10% fetal calf serum. For transfection, 3.5×10^6 washed cells were resuspended in RPMI 1640 containing $2.5 \mu\text{g}$ pDJ1.1, $7.5 \mu\text{g}$ each of RAG1 and RAG2 expression vectors (constructed by M. Gallo by inserting RAG1 or RAG2 cDNA (refs 9, 10; from D. Schatz) into the vector pCDM8) and in some cases $7.5 \mu\text{g}$ pTdt (ref. 7), an expression vector containing a murine Tdt cDNA. Transfection was by electroporation using a Bio-Rad Gene Pulser at 250 V, 960 μF . Cells were plated at 4.4×10^5 per ml.

FIG. 4 Distribution of coding joints formed by pDJ3 and by a collection of substrates. *a*, pDJ3 recombinants isolated from pre-B¹⁰. Sequence data from 43 junctions was compiled. *b*, pDJ3 recombinants isolated from pre-B^{hi}. Sequence data from 25 junctions are displayed. *c*, A compilation of data from 250 coding joint sequences from Figs 2*b* (4 maximum homology coding joints are not shown), 3*b* (2 maximum homology coding joints excluded), 4*a* and *b*, (ref. 6), and 5 additional substrates (R.M.G., manuscript in preparation). Data from *a* and *b* were compared using the Kolmogorov-Smirnov test and were not significantly different ($P=0.181$).



co-transfected with pTdt were maximum-homology coding joints (5%). This directly indicates that, when Tdt is present, homology at coding ends is not frequently used in coding joint resolution.

A second substrate with four nucleotides of homology (CCTG) at the coding ends, pJ3, was introduced into fibroblasts. Examination of independent junctions revealed 10 of 32 NP⁻ (31%) were maximum homology coding joints (data not shown). Thus we have confirmed that another homology block can dictate coding end products, indicating that CTAC is not a unique coding end. It remains to be determined whether any block of homology functions in the same way.

We have also examined recombinants from a substrate, pDJ3, that contains DFL16.1 and JH3, which have less homology at the coding ends (Fig. 1). Nine per cent of pDJ3 coding joints formed in pre-B¹⁰ (4 of 23) and 12% of those formed in pre-B^{hi} were -4 coding joints (3 of 25) (Fig. 4). Examination of 250 coding joints (Fig. 4), together with the experiments shown in Figs 2*b* and 3*b*, indicates that there is no intrinsic bias to form -4 junctions. Hence, homology is the only basis for this bias in pDJ1 and pDJ1.1.

Although one or two nucleotides of homology are present at 46% of the NP⁻ pDJ3 junctions (loss of 1, 2 or 4 nucleotides), the diverse array of coding joints among pDJ3 recombinants shows that one or two nucleotides of homology is not sufficient to constrain DJ joining substantially.

The efficiency of recombination, as determined by the ratio of recombined substrate molecules to total molecules recovered¹³, is very similar when we compare pDJ1 with pDJ3 (data not shown). This indicates that homology does not affect efficiency of the reaction, just the diversity of the products.

We have shown that when homology at coding ends is used during $V(D)J$ recombination in the absence of Tdt, the diversity of coding joints dramatically decreases. Because Tdt-mediated N-region insertion is very rare at DJ joints formed early in murine ontogeny^{1-5,14,15}, our results suggest that homology accounts for overrepresentation of certain DJH combinations, as suggested previously^{1,2}. They also show that for other DJH pairs, such as DFL16.1 and JH3, $V(D)J$ recombination is not biased.

Several $V(D)J$ loci have been proposed to use homology to form junctions, including $\gamma\delta$ T-cell receptors^{1,8}. Immunoglobulin heavy-chain *D* and *J* loci have the most potential homologies, as three of the four JHs in mouse have (C)TAC at or close to the 5' end, and 10 DHs end in (CT)AC. Also, generation of P-nucleotides may sometimes extend these homologies, as is the case for the DFL16.1-JH1 junction⁴ and $\gamma\delta$ -cell receptors⁸. The contribution of P-nucleotides to the formation of homology joints remains to be established. The generation of both single-stranded DNA overhangs that reveal homology at coding ends and P-nucleotides can be accounted for by an asymmetric opening of a hairpin intermediate^{16,17}.

By generating a high proportion of productive DFL16.1-JH1 junctions, the frequent generation of important specificities is ensured. The DFL16.1-JH1 maximum homology joint is present in most T15-positive antibodies¹⁸⁻²¹. As T15 antibodies are optimally protective against infection with *Streptococcus pneumoniae*²², restricting DJH diversity seems to be one strategy to ensure protection against this and other phosphocholine-bearing pathogens²³⁻²⁵. Perhaps the diversity-increasing benefit of Tdt outweighs the conservative trend of diversity restriction only after the establishment of the first layer of immune-specific recognition²⁶. □

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- Gu, H., Forster, I. & Rajewsky, K. *EMBO J.* **9**, 2133-2140 (1990).
- Gu, H., Kitamura, D. & Rajewsky, K. *Cell* **65**, 47-54 (1991).
- Feeney, A. J. *J. Immunol.* **147**, 4343-4350 (1991).
- Feeney, A. J. *J. Immunol.* **149**, 222-229 (1992).
- Chang, Y., Paige, C. J. & Wu, G. E. *EMBO J.* **11**, 1891-1899 (1992).
- Lieber, M. R., Hesse, J. E., Mizuuchi, K. & Gellert, M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8588-8592 (1988).
- Kallenbach, S., Doyen, N., D'Andon, M. F. & Rougeon, F. *Proc. natn. Acad. Sci. U.S.A.* **89**, 2799-2803 (1992).
- Lafaille, J. J., DeCloux, A., Bonneville, M., Takegaki, Y. & Tonegawa, S. *Cell* **59**, 859-870 (1989).
- Schatz, D. G., Oettinger, M. A. & Baltimore, D. *Cell* **59**, 1035-1048 (1989).
- Oettinger, M. A., Schatz, D. G., Gorka, C. & Baltimore, D. *Science* **248**, 1517-1523 (1990).
- Pergola, F., Zdzienicka, M. Z. & Lieber, M. R. *Molec. cell. Biol.* (in the press).
- Hesse, J. E., Lieber, M. R., Mizuuchi, K. & Gellert, M. *Genes Dev.* **3**, 1053-1061 (1989).
- Hesse, J. E., Lieber, M. R., Gellert, M. & Mizuuchi, K. *Cell* **49**, 775-783 (1987).
- Meek, K. *Science* **250**, 820-823 (1990).
- Feeney, A. J. *J. exp. Med.* **172**, 1377-1390 (1990).
- Lieber, M. R. *FASEB J.* **5**, 2934-2944 (1991).
- Roth, D. B., Menetski, J. P., Nakajima, P. B., Bosma, M. J. & Gellert, M. *Cell* **70**, 983-991 (1992).
- Gearhart, P. J., Johnson, N. D., Douglas, R. & Hood, L. *Nature* **291**, 29-34 (1981).
- Clafin, J. L. & Berry, J. *J. Immunol.* **141**, 4012-4019 (1988).

- Feeney, A. J., Clarke, S. H. & Mosier, D. E. *J. Immunol.* **141**, 1267-1272 (1988).
- Pollok, B. A., Kearney, J. F., Vakili, M. & Perry, R. P. *Nature* **311**, 376-379 (1984).
- Briles, D. E., Forman, C., Hudak, S. & Clafin, J. L. *J. exp. Med.* **156**, 1177-1185 (1982).
- Potter, M. *Ann. N.Y. Acad. Sci.* **190**, 306-321 (1971).
- Clafin, J. L., Berry, J., Flaherty, D. & Dunnick, W. *J. Immunol.* **138**, 3060-3068 (1987).
- Lai, R. B., Paranjape, R. S., Briles, D. E., Nutman, T. B. & Ottens, E. A. *J. Immunol.* **138**, 3454-3460 (1987).
- Herzenberg, L. A. & Herzenberg, L. A. *Cell* **59**, 953-954 (1989).
- Gauss, G. H. & Lieber, M. R. *Genes Dev.* **6**, 1553-1561 (1992).
- Alt, F. *et al.* *EMBO J.* **3**, 1209-1219 (1984).
- Kao, F.-T., Chasin, L. & Puck, T. T. *Proc. natn. Acad. Sci. U.S.A.* **64**, 1284-1291 (1969).

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Activation of complement by an IgG molecule without a genetic hinge

Ole Henrik Brekke* Terje E. Michaelsen†, Randi Sandin† & Inger Sandlie*‡

* Department of Biology, University of Oslo, PO Box 1050, 0316 Blindern, Norway

† National Institute of Public Health, Geitmyrsveien 75, 0462 Oslo, Norway

‡ To whom correspondence should be addressed.

THE hinge region links the two Fab arms to the Fc portion of the IgG molecule. It mediates flexibility to the molecule and serves as a connecting structure between the two heavy chains. In addition it provides space between the Fab and Fc parts. All three properties have been proposed to be important for the ability of IgG to initiate complement activation leading to complement-mediated cell lysis (CML)¹. Here we report the construction of a hinge-deleted mouse-human chimaeric IgG3 molecule with specificity for the hapten NIP (3-iodo-4-hydroxy-5-nitrophenacetyl), HM-1. HM-1 lacks the genetic hinge, but has an introduced cysteine between Ala 231 (EU numbering) and Pro 232 in the lower hinge encoded by the C_H2 exon. The introduced cysteine forms a disulphide bond between the two heavy chains of the molecule. In CML, HM-1 shows a greater activity than IgG3 wild type. This is the first time an IgG molecule without a genetic hinge has been found to be active in CML. We conclude that the hinge functioning as a spacer is not a prerequisite for complement activation. Rather, its major role seems to be to connect the heavy chains to each other in the amino-terminal part of C_H2. Because HM-1 is expected to have low Fab-Fc flexibility, this molecular feature is probably of no importance for complement activation.

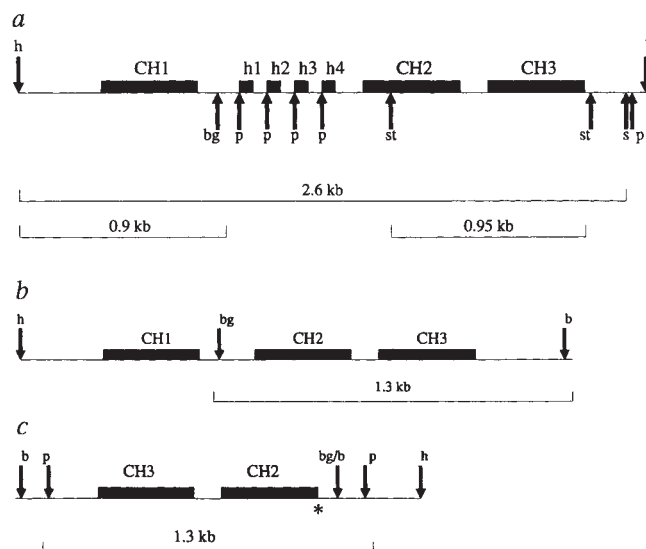
We have produced a hinge-deleted IgG3 molecule, m0, by

removing all four hinge exons from the human Cy3 gene (Fig. 1). This molecule has no disulphide bonds between the heavy chains (Fig. 2a). On a nonreducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel a large proportion of m0 molecules run as heavy-light (H-L) half molecules (Fig. 3a). In the mutant HM-1 (Fig. 2b), a cysteine was introduced between Ala 231 and Pro 232 in m0 by *in vitro* mutagenesis. SDS-PAGE analysis of HM-1 showed no presence of H-L half molecules (Fig. 3a). As m0 and HM-1 are identical except for the introduced cysteine, it is likely that the heavy chains are connected through a disulphide bond in this position. HM-1 was active in CML, and even more active than IgG3 wild type under different antigen concentrations and antigen patchiness, whereas m0 lacked CML activity completely (Fig. 4). The mutant m15, which was made by deleting three hinge exons from the Cy3 gene and therefore contains a hinge region of 15 amino acids encoded by one remaining hinge exon only², showed CML activity comparable to HM-1 (Fig. 4). In these CML assays, antigen was introduced to the target cell surface, varying both the antigen concentration and antigen patchiness. Regardless of whether the target cells had a low, medium or high antigen concentration and antigen patchiness, both HM-1 and m15 initiated lysis more effectively than normal IgG3.

Naturally occurring IgG molecules with rigid and short hinges show reduced complement activation ability³. It has been suggested that this phenomenon is due to the two Fab arms covering the binding site for the first component of the complement system, C1q, on the C_H2 domain of Fc¹, involving the amino acids Glu 318, Lys 320 and Lys 322⁴. Segmental flexibility between Fab arms and Fc (reviewed in ref. 5) depends on the length of the upper hinge³ which stretches from the end of the C_H1 to the first inter-heavy-chain disulphide bond in the hinge region⁶. We and others^{2,7-9} have constructed artificial chimaeric mouse-human IgGs where the hinge region of active

FIG. 1 Restriction maps of immunoglobulin gene constructs. *a*, Restriction map of the human Cy3 gene. Exons are shown as boxes, h, *Hind*III, bg, *Bgl*II, p, *Pst*I, st, *Sty*I, s, *Sph*I and b, *Bam*HI. *Pst*I and *Bam*HI sites outside the 2.6 kilobase (kb) gene construct resides in the pUC19 polylinker. *b*, The m0 gene construct. *c*, C_H1-C_H3 fragment as it is inserted into the M13mp18 polylinker. An asterisk marks the site of the mutation.

METHODS. A 2.6-kb *Hind*III-*Sph*I fragment containing the full-length gene of human Cy3 (G3m(b°) allotype) (a) was subcloned into pUC19². The Cy3 gene was partially digested with *Pst*I. The resulting gene construct obtained, m15, contained only the h4 hinge exon. m15 was linearized by digestion with *Bgl*II. The linearized plasmid was then subjected to exonuclease III and SI nuclease digestion. After a Klenow end-filling reaction, *Bgl*II linkers were added before ligation. A plasmid that had thereby lost its h4 exon had also lost part of the C_H1 exon. The gene construct was digested with *Hind*III and *Bgl*II and combined with a complete C_H1 exon on a *Hind*III-*Bgl*II fragment to give the m0 gene construct (b). The template for the mutagenesis was a 1.3kb *Bam*HI-*Bgl*II fragment containing the C_H3 and C_H2 exons. This fragment was subcloned into the *Bam*HI site of the polylinker of M13mp18 (c). *In vitro* mutagenesis was as described¹⁹. The mutagenesis oligonucleotide primer was as follows: 5'catctctctcagcctgcctgaactcc3'. Nucleotides coding for cysteine (underlined) were thus inserted into the C_H2 exon and the mutant sequence was verified by automatic DNA sequencing by the Sanger dideoxy chain-termination method²⁰. Only the sequenced part of the C_H2 exon encoding the N-terminal region was used. The rest of the C_H2-C_H3 fragment was exchanged with a wild-type C_H2-C_H3 *Sty*I-fragment of 950 base pairs (bp) (a). The mutant 1.3-kb C_H2-C_H3 *Pst*I fragment (c), was then cloned into a pUC19 vector containing the 0.9-kb C_H1 *Hind*III-*Pst*I fragment. The mutant Cy3 gene construct was finally cloned into the pSV2gptV_{NP} shuttle vector²¹ as a 2.2-kb



*Hind*III-*Bam*HI fragment. Transfection was done by electroporation of J558L cells as described^{7,22}. The V_H segment of the pSV2gpt vector expresses the V_H-region characteristic of a λ₁ light-chain-bearing mouse antibody that recognizes the hapten NIP²³. J558L does not produce an immunoglobulin heavy chain but produces a λ₁ light chain. The mutant chimaeric antibodies produced by the transfectomas thus have specificity for the hapten NIP. Clones were selected in medium containing mycophenolic acid and xanthine, screened for antibody production by ELISA² and subjected to limiting dilution.

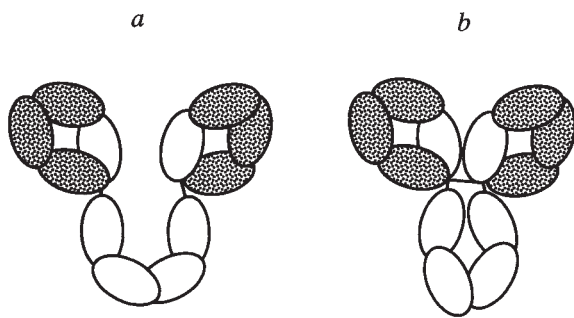


FIG. 2 Models of chimaeric mouse-human mutant IgG. *a*, Model of m0 produced as H-L half molecules. Shaded domains are murine and open domains are human. The heavy chains are not connected as the genetic hinge is deleted. The resulting conformation of the molecule is that of an open structure as there are noncovalent interactions between C_{H3} domains⁷. *b*, Model of HM-1. The heavy chains are covalently linked by a disulphide bond provided by the inserted cysteine as the second amino acid of the lower hinge.

IgG molecules has been made short and rigid by removing amino acids in the N-terminal region. These molecules lost none of their complement activation potential. Thus, segmental flexibility has been shown to be of no importance for CML. But the hinge region seems to be necessary for complement activation because all IgG mutants without the genetic hinge so far are inactive^{7,10,11}. The middle, or core hinge, contains cysteines mediating disulphide bonds between the heavy chains and a rigid polyproline core which separates Fab from Fc^{12,13}. In the Dob and Mcg molecules, both without genetic hinge, the light chains are disulphide-bonded to each other, and the crystal structures of these molecules show that the distance between Fab and the C_{H2} domains is very short^{14,15}. It has therefore been proposed that the hinge may serve to create space between the Fabs and the Fc part of the molecule, allowing Clq to interact with its binding site on C_{H2} . Because HM-1 lacks the genetic hinge region, the distance between the Fab arms and Fc must be short. Therefore, it seems likely that the ability of the hinge to provide space between the Fab arms and Fc is not necessary for complement activation. Neither does HM-1 have an upper hinge, as there is only a single amino acid between the end of C_{H1} and the first inter-heavy-chain disulphide bond, namely Ala

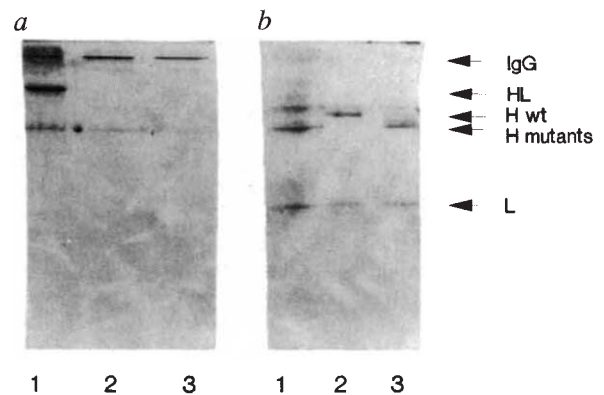


FIG. 3 SDS-PAGE gel (12%). The lanes contain: 1, m0 mutant; 2, IgG3 wild type; and 3, HM-1 mutant. *a*, Without dithiothreitol (DTT) treatment. *b*, Treated with 10mM D/T. IgG, H_2L_2 ; HL, heavy and light chain; H wt, wild type heavy chain monomers; H mutants, mutant heavy-chain monomers; L, light-chain monomers.

METHODS. Antibodies were isolated from 1 litre of cell culture supernatants by affinity chromatography on a NIP-Sepharose column and eluted with the hapten NIP as described², then analysed by SDS-PAGE on a 12% gel as described²⁴.

231. This amino acid belongs to the lower hinge encoded by the C_{H2} exon. Segmental flexibility between the Fab arms and Fc is therefore not necessary for complement activation. Rather we observed that the presumably inflexible HM-1 molecule activated complement better than the corresponding IgG3 wild-type molecule regardless of antigen concentration and antigen patchiness. We also observed this phenomenon when testing m15, which has a short and rigid hinge^{7,16}. It seems as though the long and flexible hinge of IgG3 wild type reduces its CML potential. Clq binding to rigid molecules may be energetically favourable compared to the flexible IgG3 molecule, as the loss of entropy on complex formation is greater for a flexible molecule than for a rigid one.

Our mutant HM-1 and the proteins Dob and Mcg all consist of two heavy chains and two light chains (H_2L_2). But unlike HM-1, both Dob and Mcg have the two H-L half molecules disulphide-bonded through linking of the light chains^{15,14} and neither can complement fixate^{10,11}. Because the HM-1 molecule has CML activity, there must be conformational differences between these proteins regarding access to the Clq binding site. A clue to

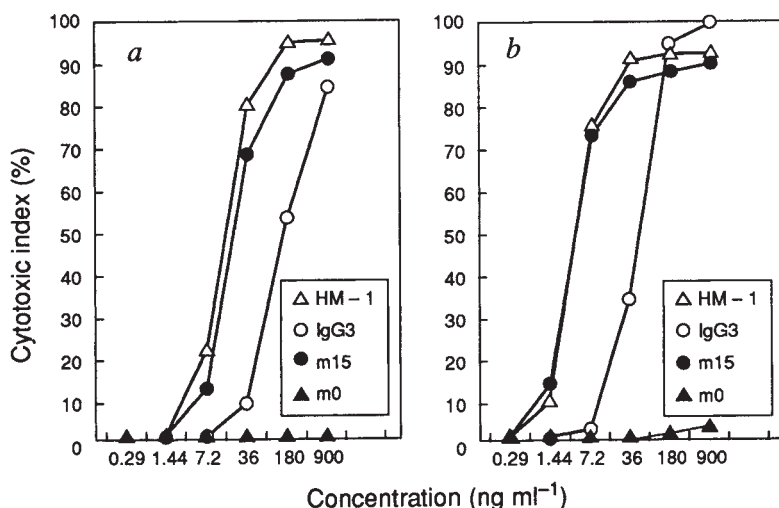


FIG. 4 CML activity induced by NIP-specific antibodies measured in a ^{51}Cr -release assay. The target cells were sheep red blood cells (SRBC), and the hapten NIP was introduced on the cell surface conjugated to anti-SRBC Fab fragments. For 1×10^8 SRBC a total of either: *a*, low amount, 80 ng NIP with NIP₄Fab; or *b*, high amount, 2,000 ng NIP with NIP₆₀Fab. HM-1, hinge-deleted IgG3 with an introduced cysteine; m0, hinge-deleted IgG3; m15, IgG3 with genetic hinge of 15 amino acids; IgG3, IgG3 wild type.

METHODS. The CML assay was done by varying antigen concentration and antigen patchiness as described previously⁷. Briefly, target cells, ^{51}Cr -SRBC, were allowed to react with NIP-conjugated rabbit anti-SRBC Fab' fragments. Serial dilutions of chimaeric antibodies were then added to the target cell suspension and human serum was used as the complement source. The cytotoxic index (CI) was calculated according to the formula:

$$\% \text{ CI} = \frac{\text{c.p.m. (test)} - \text{c.p.m. (spontaneous)}}{\text{c.p.m. (max)} - \text{c.p.m. (spontaneous)}} \times 100$$

these differences is probably the disulphide bond between the light chains in Dob and Mcg, which is not present in the HM-1 molecule.

Hinge-deleted molecules such as m0, lack disulphide bonds between the heavy chains. The C_H2 domains will therefore drift apart because there are no transinteractions between these domains^{17,18} (Fig. 2a). As a result, the Clq-binding sites on the two C_H2 domains will be dislocated and may even be distorted. Because Clq is multivalent in its binding to IgG, tight cooperative binding between IgG and Clq may be impossible for the m0 molecule. The only difference between m0 and HM-1 is in the ability of HM-1 to form a disulphide bond between its heavy chains. Thus in complement activation the major role of the hinge seems to be covalent linking of the heavy chains.

It has been difficult to crystallize intact active IgG molecules for X-ray diffraction analysis because of the flexible hinge region. The mutant HM-1 is likely to be rigid, therefore it may be a good candidate for X-ray analysis of an intact, functionally active IgG molecule.

Note added in proof: We have recently shown that HM-1 promotes phagocytosis mediated by FcRI with high efficiency, but m0 is negative²⁵. Thus, bridging of the heavy chains is also a prerequisite for efficient FcR signalling, whereas Fab arm flexibility and distance between Fab and Fc seem not to be necessary. □

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1. Feinstein, A., Richardson, N. & Taussig, M. J. *Immun. Today* **7**, 169–174 (1986).
2. Sandlie, I., Aase, A., Westby, C. & Michaelsen, T. E. *Eur. J. Immun.* **19**, 1599–1603 (1989).
3. Dangl, J. L. et al. *EMBO J.* **7**, 1989–1994 (1988).
4. Duncan, A. R. & Winter, G. *Nature* **332**, 738–740 (1988).
5. Nezlín, R. *Adv. Immun.* **48**, 1–40 (1990).
6. Burton, D. R. *Molec. Immun.* **22**, 161–206 (1985).
7. Michaelsen, T. E., Aase, A., Westby, C. & Sandlie, I. *Scand. J. Immun.* **32**, 517–528 (1990).
8. Norderhaug, L. et al. *Eur. J. Immun.* **21**, 2379–2384 (1991).
9. Tan, L. K., Shopes, R. J., Oi, V. T. & Morrison, S. L. *Proc. natn. Acad. Sci. U.S.A.* **87**, 162–166 (1990).
10. Klein, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **78**, 524–528 (1981).
11. Deutsch, H. F. & Suzuki, T. J. *Immun.* **107**, 929–930 (1971).
12. Marquart, M., Deisenhofer, J. & Huber, R. *J. molec. Biol.* **141**, 369–391 (1980).
13. Ito, W. & Arata, Y. *Biochemistry* **24**, 6467–6474 (1985).
14. Rajan, S. S. et al. *Molec. Immun.* **20**, 787–899 (1983).
15. Silverton, E. W., Navia, M. A. & Davies, D. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5140–5144 (1977).
16. Schneider, W. P., Wensel, T. G., Stryer, L. & Oi, V. T. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2509–2513 (1988).
17. Michaelsen, T. E. *Scand. J. Immun.* **5**, 1123–1128 (1976).
18. Seegan, G. W. et al. *Proc. natn. Acad. Sci. U.S.A.* **76**, 907–911 (1979).
19. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. *Meth. Enzym.* **154**, 367–382 (1987).
20. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
21. Neuberger, M. S. et al. *Nature* **314**, 268–270 (1985).
22. Potter, H., Weir, L. & Leder, P. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7161–7165 (1984).
23. Neuberger, M. S. *EMBO J.* **2**, 1373–1378 (1983).
24. Laemmli, U. K. *Nature* **227**, 680–685 (1970).
25. Aase, A., Sandlie, I., Norderhaug, L., Brekke, O. H. & Michaelsen, T. E. *Eur. J. Immunol.* (in the press).

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Segmental organization of embryonic diencephalon

Michael C. Figgdor*† & Claudio D. Stern‡§

* Institute of Histology and Embryology, Schwarzschanerstrasse 17, 1090 Vienna, Austria

‡ Department of Human Anatomy, University of Oxford, South Parks Road, Oxford OX1 3QX, UK

† Present address: Beckman Institute, California Institute of Technology, Pasadena, California 91125, USA.

THE diencephalon is a complex integration centre and intricate relay station of the vertebrate brain^{1–3}. Its development involves the generation of great cellular diversity and neuronal specificity. We report here that it becomes organized in steps, through a stereotyped sequence of neuromeric subdivisions. Diencephalic neuromeres define four cellular domains (D1–D4) that can be followed throughout development, each unit contributing to a well defined part of the adult structural pattern. We propose that the segmental identity of each diencephalic unit is specified by a unique combination of genes^{4–13}, maintained by polyclonal cell lineage restrictions. A comparison of vertebrate and arthropod development suggests that the basic principles that control anterior axial patterning and set up neuronal specificity in the embryonic central nervous system are highly conserved in evolution.

Many morphological studies have been done^{14–21} to determine the basic units of pattern formation in the forebrain, but they have come to irreconcilable conclusions. We have now studied the development of the embryonic diencephalon of the chick embryo by scanning electron microscopy (SEM). A pattern of transverse furrows and ridges is seen, repeated along the anteroposterior axis of the third ventricle (Fig. 1a). Four regions can be distinguished, which we designate as neuromeres D1–D4, numbered in anterior-to-posterior sequence. The borders between neuromeres appear as ridges, subdividing the otherwise smooth relief of the third ventricle. They form in precise temporal sequence (D4/M: stage-12 (ref. 22); D1/D2:

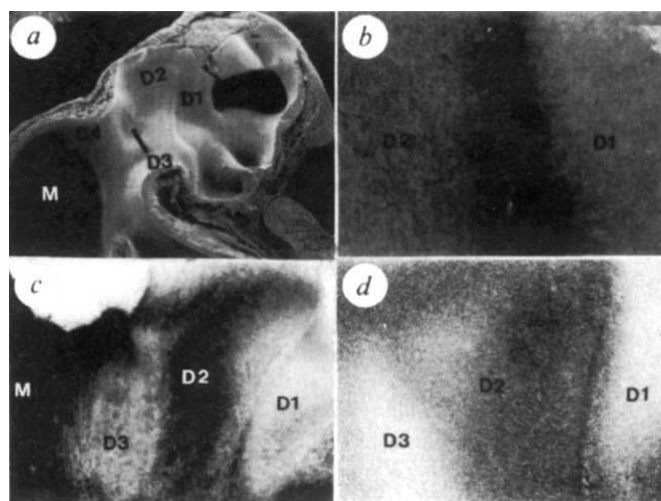


FIG. 1 Morphology of diencephalic neuromeres and alternating patterns revealed by antibodies. *a*, SEM of stage-24²² chick embryo in medial view. *b*, NCAM is accumulated at neuromere boundaries: the border between D1 and D2 stains intensely. Whole mount of stage-24²² embryo. Acetylcholinesterase activity (*c*) and peanut agglutinin (*d*) reveal an alternating pattern of staining: even-numbered neuromeres show high levels of activity. In D4, the development of the early tract of the posterior commissure (TPC axons) is seen (*c*), restricted to both anterior and posterior boundaries of D4. Whole mount of stage-25²² embryo. D1–D4, diencephalic neuromeres. T, telencephalon. M, mesencephalon.

METHODS. For SEM, chick embryos at stages 12–39²² were explanted in PBS, pH 7.4 at 4°C and the brain bisected. They were fixed for 1–3 h in 2% paraformaldehyde, 2.5% glutaraldehyde and 0.025% CaCl₂ in 0.1 M cacodylate buffer (pH 7.4). After several washes in this buffer, they were incubated for 1 h in 2% OsO₄, washed in cacodylate buffer, dehydrated, transferred to acetone and critical-point dried overnight. Specimens were mounted on stubs, sputter coated (Nanotech SEM Prep 2, 4 min) and viewed with a Philips SEM 515. Antibody, cholinesterase and lectin histochemistry were done by previously described techniques^{23–25}. Anti-NCAM antibody was a gift of Dr U. Rutishauser.

§ To whom correspondence should be addressed.

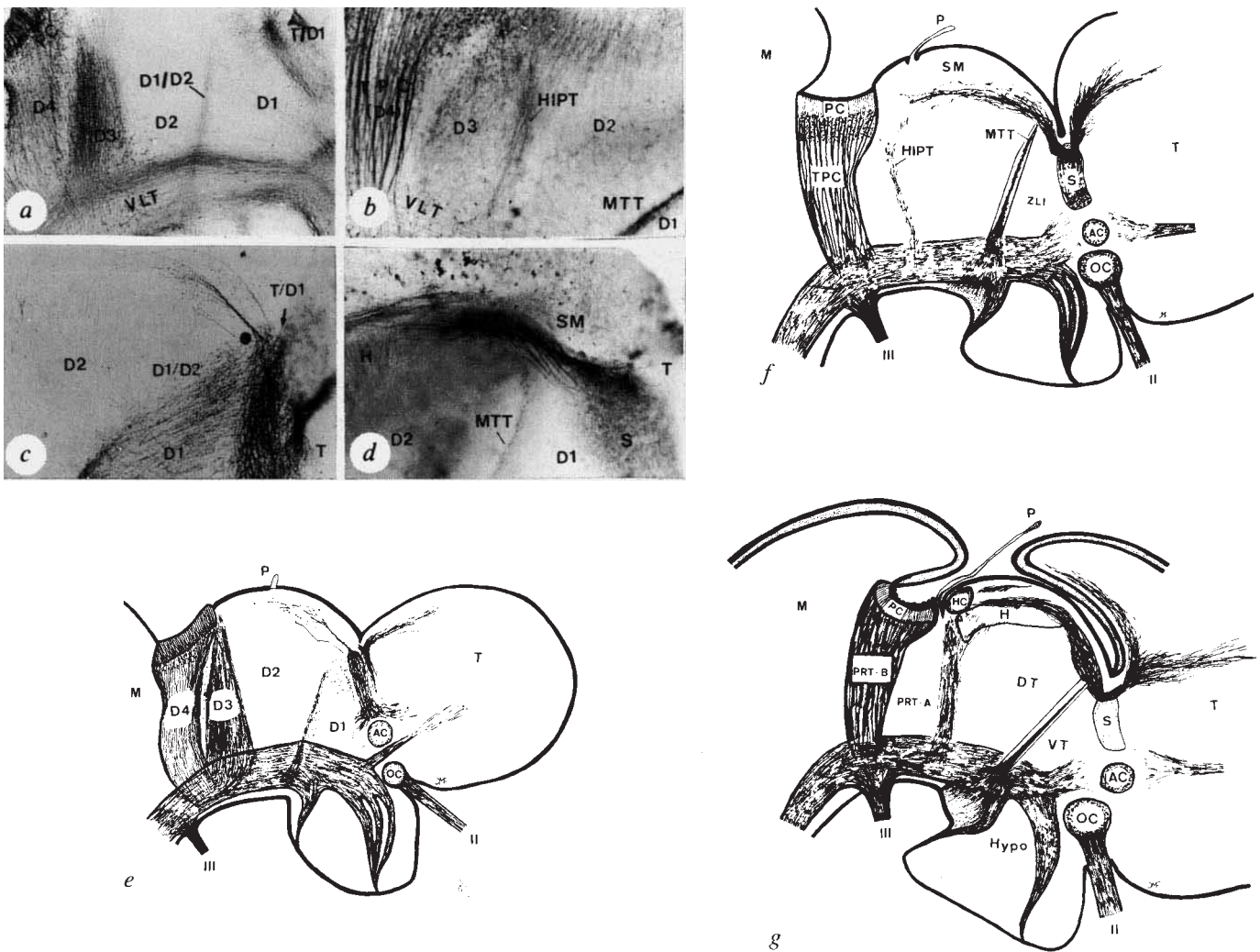


FIG. 2 Development of the segmental scaffold of primary axon fascicles in the embryonic chick diencephalon, visualized in whole mounts with an antibody against neurofilament-associated proteins³⁷. *a*, Stage-27²². D4 can be defined by the formation of the posterior commissure (PC) and its tract (TPC). D3 contains a population of axons, constrained by both boundaries of D3. D2 is limited anteriorly by an axon fascicle, the presumptive mammillothalamic tract (MTT), developing in the ZLI. *b*, The D1/D2 pathway forms a barrier respected by D1 axons. Stage 25. Individual D1 axons change their direction locally by 90° along the T/D1 border, starting to fasciculate with each other at the D1/D2 border. A small set of primary stria medullaris (SM)-axons changes its direction abruptly at the dorsal margin of the T/D1 border (circle). Leading SM-axons seem to pioneer as individuals, forming a fan of very fine axonal processes. *c*, *d*, The adult organization of diencephalic connectivity develops from the primary neuromeric scaffold. Stage 40²². The pattern of major

diencephalic axon tracts and commissures forms by addition of a large number of axons onto the primary axonal scaffold. D4 can be defined by the selective fasciculation of TPC-axons to form thick cables, characteristic of the adult. Anteriorly (*d*), the septal region (S) can be seen to develop from the early T/D1 border. D2 axons originating from the habenula (H) grow posteriorly, forming the D2/D3 fascicle (HIPT, *c*). *e*, Stage-27²²; *f*, stage-30²²; *g*, stage-40²². The pineal (P), arising anterodorsally in D2, gradually shifts posteriorly to the point where the D2/D3 and D3/D4 borders abut, thus separating the habenular commissure (HC) from PC. II, Optic nerve; III, exit point of oculomotor nerve; AC, anterior commissure; OC, optic chiasm and associated commissures; DT, dorsal thalamus; VT, ventral thalamus; Hypo, hypothalamus; PRT-A, -B, anterior and posterior parts of pretectum. METHODS. Binding of monoclonal antibody 3A10³⁷ was visualized by immunoperoxidase histochemistry as described in Fig. 1.

stage-14 (ref. 22); T/D1: stage-15 (ref. 22); D3/D4: stage-16 (ref. 22); D2/D3: stage-17 (ref. 22) and have characteristic shapes and sizes: even-numbered neuromeres are broad dorsally, odd neuromeres are broad ventrally.

Localization of the neural cell adhesion molecule (NCAM), acetylcholinesterase (AChE) activity and peanut agglutinin (PNA) binding demonstrates differences in the morphological pattern: NCAM is concentrated at the D1/D2 boundary (Fig. 1*b*). AChE (Fig. 1*c*) and PNA-binding (Fig. 1*d*) reveal an alternating pattern: the reactions are intense in even-numbered neuromeres and weak in odd-numbered ones. These patterns are interesting because in other embryonic regions (somite halves²³, rhombomeres^{24,25} and barrel fields in the soma-

tensory cortex²⁶) NCAM, cholinesterase and PNA-receptors may be used to define morphogenetic units.

How does this simple metamer pattern correspond to the complex organization of the adult diencephalon? Following the pattern of axonal differentiation continuously during development demonstrates that the basic organization of diencephalic axonal connectivity is established by stereotypic addition of large numbers of axons onto a simple grid of primary fascicles. A direct correspondence is seen between the early neuromeric pattern and the adult structural pattern (Fig. 2).

A small number of embryonic axons appears at invariant positions within the embryonic chick forebrain, using neuromere boundaries to navigate, forming a well defined set of adult

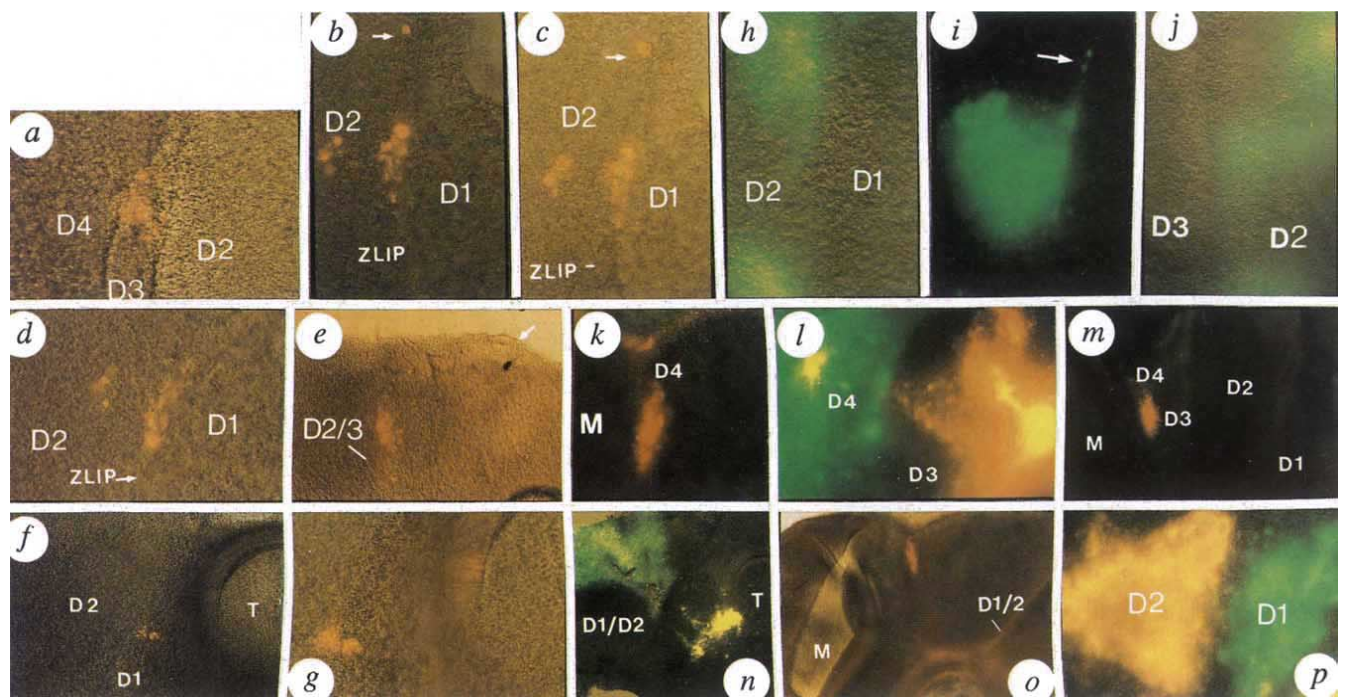


FIG. 3 Neuromere boundaries represent borders of polyclonal lineage restriction, demonstrated by intracellular injection of lysine-rhodamine-dextran (LRD) into single cells (a–g) and by labelling with carbocyanine dyes (h–p). a–g, Results from single cell injection of LRD into 318 stages 15–21²² embryos. Clones varied in size from a few cells to up to 600 and spanned up to 300 μm . At stage-17²², D1 and D3 measure 150 μm at their longest point, D2 extends over 500 μm , and D4 is 250 μm long. From the extent of the clones in each neuromere we calculated the probability that the lineage restrictions are due to chance. In injections before stage-10, clones frequently cross neuromere boundaries ($\chi^2=2.03$, 1 d.f.; $P>0.1$). By contrast, clones generated after stage-17 never cross ($\chi^2=19.0$, 1 d.f.; $P<0.001$). a, 32-cell clone (labelled at stage-19²²), restricted at both boundaries of D3, spanning the whole width of the neuromere. b, c, Part of this 26-cell clone (injected stage-18²²) is located within the D1/D2 boundary. Despite extensive dorsoventral spread (one cell (arrow) can be seen nearly at the dorsal midline), the clone does not enter D1. The photographs are taken at different focal planes. d, 60-cell clone (injected at stage-16²²) aligned along D1/D2 boundary. e, >200-cell, D-shaped clone at the D2/D3 boundary (stage-18²² injection). Arrow, pineal gland. f, g, Stage-15²² injection. Labelled cells are in two main groups, each restricted by one of the boundaries of D1. h, p, Labelled cells resulting from 279 focal injections of carbocyanine dyes (Dil, red and DiO, green) into the forebrain (stages 14–23²²). h, Labelled cells restricted at the D1/D2 boundary. i, Both boundaries of D2 are respected, and there is extensive spread of

labelled cells along the D1/D2 boundary (arrow). j, Labelled progeny of an injection at stage-16²², restricted at the D2/D3 boundary. k, Dil injection targeted at the D4/M border; labelled cells almost fill the neuromere but do not cross into either D3 or the midbrain. l, Injection of Dil (D3) and DiO (D4), showing considerable spread within each neuromere but neither crosses the boundary. The D4 group aligns along the border. m, Dil injection (stage-18²²). Labelled cells are restricted to D4. n, Dil injection into the anterior forebrain and DiO into posterior forebrain (early stage-14²² embryo), showing considerable spread within D1, with a few cells reaching the D1/D2 border. In the posterior forebrain, DiO-labelled cells are only restricted at the D1/D2 boundary; they spread extensively posterior to this border. At stage-14²², only the D1/D2 and D4/M boundaries have formed. Labelled cells have therefore spread across the T/D11 (Dil) and D2/D3 (DiO) boundaries. o, Two separate Dil injections (stage-18²²): the posterior one gave rise to cells restricted to D4. The anterior injection generated labelled primary axons at the D1/D2 boundary. p, Dil injection into D1 and DiO into D2 (stage-16²²). Labelled cells reach the D1/D2 border but do not intermix.

METHODS. LRD^{29–30} (10 mg ml⁻¹) was injected *in ovo*, essentially as described previously^{29,30}. Filled electrode impedance was 60–100 M Ω . Embryos were fixed after 36–48 h reincubation. 3,3'-Diocetadecyl indocarbocyanine perchlorate (Dil) or 3,3'-diocetadecyloxycarbocyanine perchlorate (DiO) (Molecular Probes) were injected by air pressure *in ovo*. Embryos were fixed after 48–72 h incubation.

axon tracts and commissures that is highly conserved during vertebrate evolution^{1–3,14,15}, each structural element separating the major diencephalic regions of the adult. Diencephalic neuromere borders therefore appear to be a conserved scaffold of pre-existing, region-specific axon pathways in the early forebrain neuroepithelium. This is similar to the development of the central nervous system (CNS) in arthropods, which is established on a simple segmental plan^{27,28}: the growth cones of early pioneer neurons of insects use positional cues within the neuroepithelium, making cell-specific choices at local decision regions to set up a stereotyped, orthogonal scaffold of axon fascicles, repeated at each embryonic segment. Later-developing axons grow along this basic framework, each choosing uniquely 'labelled' axon-pathways on which they selectively fasciculate.

If segmentation of the diencephalon is important for pattern formation, then an effective mechanism must preserve the

autonomy of the individual units: unrestrained cell mixing across segment boundaries would destroy the metameric pattern. We used two different approaches to test whether diencephalic neuromere boundaries represent barriers to the mixing of cells. First, we injected a fluorescent lineage tracer^{29,30} into a single neuroepithelial cell. Clones generated from injections made before formation of neuromere borders (stages 6–12, ref. 22) often crossed into the adjacent neuromere. In contrast, all clones generated from injections made after border formation failed to spread into the adjacent neuromere (Fig. 3 a–g). In some cases, especially in D3, whose anterior and posterior borders are very close (Fig. 3a), the clone abutted two borders and respected both. Clones were unable to cross the boundaries despite their extensive spread in other directions (Fig. 3b–g) and their large size, and were often aligned along the adjacent border (Fig. 3b–e). Within the limits of individual diencephalic neuromeres, labelled and unlabelled cells were intermixed

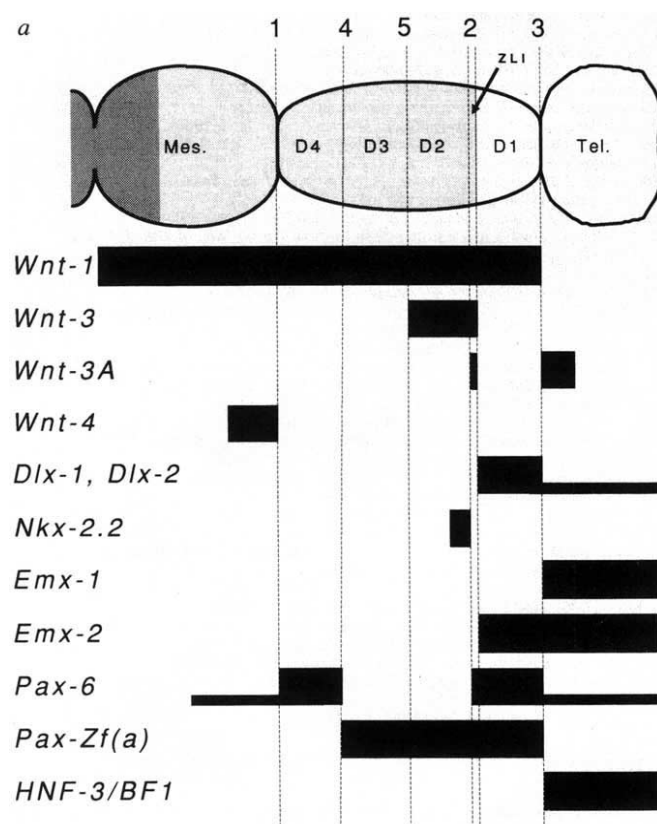


FIG. 4 *a*, Expression domains (shown as black horizontal bars) of 12 putative regulatory genes⁵⁻¹³ from mouse, zebrafish and frog that respect diencephalic neuromere boundaries. Boundaries were defined by the morphological studies described above and using the position of axon fascicles (Fig. 2) as landmarks to reinterpret the published domains of expression of these genes. In the mouse, diencephalic neuromeres appear at 9–11 days postcoitum (p.c.) and are comparable to their chick counterparts (unpublished observations). The diagram also shows the region (light shading) competent to express the *engrailed* homologue, *En-2*, on grafting of mesencephalic

(b)

Early diencephalic neuromeres and their boundaries

Neuromeres and their boundaries	Adult structures
Telencephalon/D1 (T/D1) boundary	Septum verum (S) Septum pellucidum (mammals) Anterior commissure (AC) Anterior pallial commissure (APC) (corpus callosum of mammals) Posterior pallial commissure (PPC) (hippocampal commissure) Fornix Optic chiasm (OC)
D1	Ventral thalamus (VT) Hypothalamus (Hypo)
D1/D2 boundary	Zona limitans intrathalamica (ZLI) Mammillothalamic tract (MTT)
D2	Dorsal thalamus (DT) Habenula (H) Stria medullaris (SM)
D2/D3 boundary	Habenulo-interpeduncular tract (HIPT) Habenular commissure (HC) Pineal gland (P)
D3	Anterior part of pretectum (PRT-A) (excluding PC and TPC)
D3/D4 boundary	Anterior border of posterior commissure (PC) and its associated tract (TPC)
D4	PC + TPC-containing part of pretectum (posterior part of pretectum; PRT-B)
D4/midbrain boundary (D4/M)	Posterior border of PC + TPC Anterior border of tectal commissure Exit point of oculomotor nerve (III)

neuroepithelium³⁶ and the region that normally expresses *En-2* (dark shading). The numbers above the diencephalic neuromere boundaries correspond to the order in which these borders form during development. *b*, Correspondence between early diencephalic neuromeres and their boundaries and the adult structures that form in them. This correspondence was used to relate the expression domains of individual genes to the neuromeric pattern.

extensively (Fig. 3*b–g*), showing no tendency to align or disperse in any direction. This suggests that the early embryonic diencephalon is not further subdivided by a set of hidden boundaries.

Second, we marked small groups of cells within diencephalic neuromeres, using targeted microinjections of carbocyanine dyes. This allowed us to demonstrate directly that adjacent groups of cells do not mix across an intervening neuromere boundary. The results confirm those obtained by intracellular injection (Fig. 3*h–p*): the progeny of cells labelled after border formation fails to cross any of the borders encountered. Injections often produced progeny that filled the entire neuromere with labelled cells, challenging both boundaries (Fig. 3*i,k,p*).

Both experiments demonstrate that diencephalic neuromeres represent discrete units of polyclonal lineage restriction, like other segmented structures in the vertebrate embryo (somites^{29,31}, hindbrain³⁰). In insects, holoclonal lineage restrictions have been suggested to define developmental compartments, each comprising “all the surviving descendants of a small group of founder cells, which respects defined spatial boundaries”^{32,33}. These boundaries could act to define the limits of autonomous units of pattern formation, “. . . the animal being made in modules, each growing and shaping itself independently of the others and each being subject to somewhat

independent genetic control”³³.

In both insects and vertebrates, the head appears to be subdivided into two segmented regions. Posterior (gnathal in insects, hindbrain in vertebrates) segments are specified by a combinatorial code of gene expression that continues into the trunk (Hox code³⁴). Recent results^{10,35} suggest that the anterior segments of both (cephalic in insects, forebrain/midbrain in vertebrates), which contain the higher integration centres, seem to use a fundamentally different code. The development of a highly conserved set of invariant reference points in the vertebrate forebrain, derived from the early metamereric pattern (Figs 1*a*, 2 and 4*b*) allows us to interpret the domains of gene expression described in this region. Several putative regulatory genes are restricted at diencephalic neuromere boundaries (Fig. 4).

A recent experiment in the chick embryo³⁶ demonstrated that the potential of the anterior neural tube to express *Engrailed-2* (*En-2*) and to develop mesencephalic fates stops sharply at the zona limitans intrathalamica (ZLI), which our results reveal to be derived from the earliest developing intraprosencephalic neuromere border (D1/D2). Thus, an early step in pattern formation of the vertebrate forebrain may be the segregation of two supra-segmental polyclones of founder cells, each displaying a fundamentally different cell state: an anterior one (T+D1), incompetent to express *En-2* and lacking mesencepha-

lic potential, and a posterior one (D2+D3+D4), competent to express *En-2*, and able to develop mesencephalic fates.

In *Drosophila*, interactions between cells expressing the segment polarity genes *wingless* (*wg*) and *engrailed* (*en*) are required to set up and to pattern segmental units by controlling the expression of other genes, such as the homeobox gene *distal-less* (*dll*)³⁵. In vertebrates, the border of expression of two homologues of *wg*, *Wnt-3* and *Wnt-3A* (ref. 5) not only coincides with the same diencephalic neuromere border (D1/D2;ZLI) where *En-2* competence stops, but also abuts directly with the expression domains of several members of the *Dlx* multigene family, the vertebrate homologues of *dll* (refs 7, 9). Thus, in both arthropods and vertebrates, the same genes could be used not only to pattern segmental units in anterior regions of the head, but also to set up a simple system of local positional cues and uniquely labelled pathways, required by growth cones to navigate selectively within the embryonic CNS. □

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- Ramón y Cajal, S. *Studies on the Diencephalon* (Thomas, Springfield, 1966).
- Ariens Kappers, C. U., Huber, G. C. & Crosby, E. C. *The Comparative Anatomy of the Nervous System of Vertebrates, Including Man* (Hafner, New York, 1960).
- Sarnat, H. B. & Netsky, M. G. *Evolution of the Nervous System* (Oxford Univ. Press, Oxford, 1981).
- Wilkinson, D. G., Bailes, J. A. & McMahon, A. P. *Cell* **50**, 79–88 (1987).
- Roelink, H. & Nusse, R. *Genes Dev.* **5**, 381–388 (1991).
- McGrew, L. L., Otte, A. P. & Moon, R. T. *Development* **115**, 463–473 (1992).
- Price, M. *et al.* *Nature* **351**, 748–751 (1991).
- Price, M. *et al.* *Neuron* **8**, 241–255 (1992).
- Porteus, M. H. *et al.* *Neuron* **7**, 221–229 (1991).

- Simeone, A. *et al.* *Nature* **358**, 687–690 (1992).
- Walther, C. & Gruss, P. *Development* **113**, 1435–1449 (1991).
- Krauss, S. *et al.* *Nature* **353**, 267–270 (1991).
- Tao, W. & Lai, E. *Neuron* **8**, 957–966 (1992).
- Kühlenbeck, H. *The Central Nervous System of Vertebrates* (S. Karger, Berlin, 1973).
- von Kupffer, K. in *Handbuch der vergleichenden und experimentellen Entwicklungslehre der Wirbeltiere*. (ed. Hertwig, O.). Bd 2, Teil 3 (Fischer, Jena, 1906).
- Rendahl, H. *Acta zool.* **5**, 119–344 (1924).
- Bergquist, H. *Progr. Brain Res.* **5**, 223–229 (1964).
- Vaage, S. *The Segmentation of the Primitive Neural Tube in Chick Embryos* (Gallus domesticus) (Springer, Berlin, 1969).
- Keyser, A. *Acta anat.* **83** (suppl. 59), 1–177 (1972).
- Puelles, L., Amat, J. A. & Martínez-de-la-Torre, M. J. *comp. Neurol.* **266**, 247–268 (1987).
- Altman, J. & Bayer, S. A. J. *comp. Neurol.* **275**, 346–405 (1988).
- Hamburger, V. & Hamilton, L. J. *Morph.* **88**, 49–92 (1951).
- Stern, C. D., Sisodia, S. M. & Keynes, R. J. *J. Embryol. exp. Morph.* **91**, 209–226 (1986).
- Lumsden, A. & Keynes, R. *Nature* **337**, 424–428 (1989).
- Lay, P. G. & Alber, R. *Development* **109**, 613–624 (1990).
- Cooper, N. G. F. & Steindler, D. A. J. *comp. Neurol.* **249**, 157–169 (1986).
- Bate, C. M. & Grünwald, E. B. J. *Embryol. exp. Morph.* **61**, 317–330 (1981).
- Thomas, J. B. *et al.* *Nature* **310**, 203–207 (1984).
- Stern, C. D. *et al.* *Development* **104** (suppl.), 231–244 (1988).
- Fraser, S., Keynes, R. & Lumsden, A. *Nature* **344**, 431–435 (1990).
- Stern, C. D. & Keynes, R. J. *Development* **99**, 261–272 (1987).
- García-Bellido, A., Ripoll, P. & Morata, G. *Nature New Biol.* **245**, 251–253 (1973).
- Lawrence, P. A. *Nature* **344**, 382–383 (1990).
- McGinnis, W. & Hoxiauf, R. *Cell* **68**, 283–302 (1992).
- Cohen, S. M. & Jürgens, G. *Nature* **346**, 482–485 (1991).
- Martínez, S., Wassef, M. & Alvarado-Mallart, R. M. *Neuron* **6**, 971–981 (1991).
- Yamada, T. *et al.* *Cell* **64**, 635–648 (1991).

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Protein kinase C is required for light adaptation in *Drosophila* photoreceptors

R.C. Hardie*, A. Peretz†, E. Suss-Toby†, A. Rom-Glas†, S. A. Bishop*, Z. Selinger‡ & B. Minke†§

* Department of Zoology, Cambridge University, Cambridge CB2 3EJ, UK

† Departments of Physiology and ‡ Biological Chemistry, Minerva Center for Studies of Visual Transduction, The Hebrew University, Jerusalem 91010, Israel

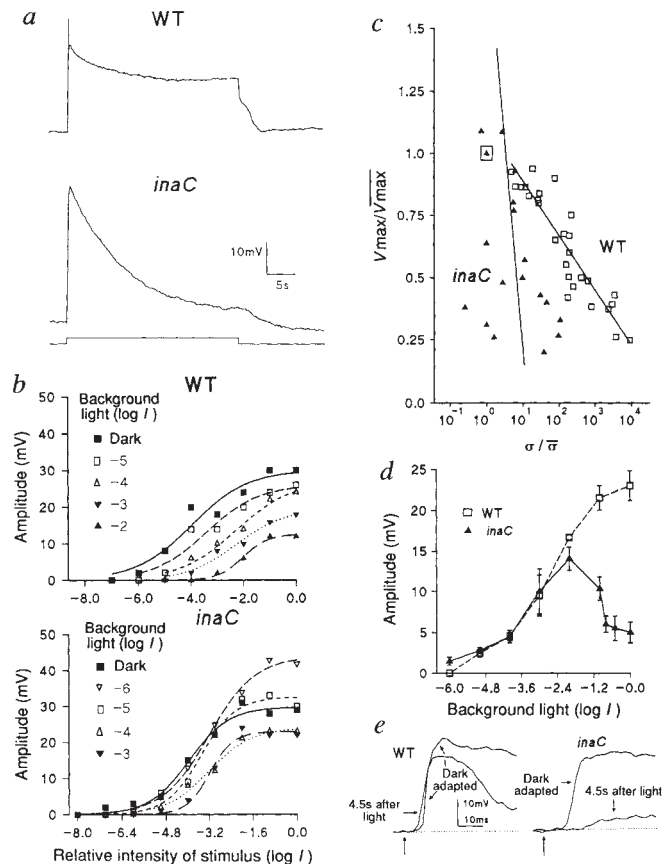
PROTEIN kinase C (PKC) is a key enzyme for many cellular processes^{1,2} but its physiological roles are poorly understood. An excellent opportunity to investigate the function of PKC has been provided by the identification of an eye-specific PKC in *Drosophila*^{3–5} and a null PKC mutant, *inaC*^{P209} (refs 5,6). Bright conditioning lights delivered to *inaC* photoreceptors lead to an abnormal loss of sensitivity in whole cell recordings from dissociated ommatidia; this has been interpreted as 'hyper-adaptation' and PKC's role has been suggested to be distinct from light adaptation⁵. A presumably related finding is that during intense light, the response of *inaC* declines to baseline⁶. Invertebrate photoreceptors use the phosphoinositide signalling cascade^{7–12}, responding to single photons with so-called quantum bumps¹³ which sum to form the macroscopic response to light^{14–16}. Light adaptation allows photoreceptors to adjust their sensitivity over the enormous range of ambient intensities^{14,17}. Although the molecular mechanism of light adaptation remains obscure, it is a negative-feedback process¹² mediated by a rise in cytosolic calcium^{12,18} and a decrease in bump size^{12,14–16}. We now show that under physiological conditions light adaptation is severely reduced in *inaC*, suggesting that eye-specific PKC, itself activated by a rise in cytosolic calcium^{4,5} and diacylglycerol, is

required for adaptation. Furthermore, we show that in the absence of PKC individual bumps fail to terminate normally, an effect that can account for the pleiotropic manifestations of the *inaC* phenotype.

Another mutant showing behaviour similar to *inaC* is the transient receptor potential mutant (*trp*). In *trp* this phenotype, which we refer to as response inactivation¹⁹, is thought to represent exhaustion of the excitatory process^{20,21}, and in fact virtually all manifestations of light adaptation are lacking in this mutant^{19–22}. The primary defect in *trp* is the absence of a light-activated Ca^{2+} channel^{23,24}, suggesting that inactivation reflects depletion of the inositol trisphosphate (InsP_3)-sensitive calcium stores and that these require Ca^{2+} influx through the *trp*-dependent channels to be rapidly refilled. The measure previously used to assess 'adaptation' in *inaC* (decrease in response amplitude)⁵ is common to both adaptation and inactivation¹⁹. Also, the inclusion of 10mM EGTA in excess of Ca^{2+} in the whole cell recording pipette⁵ and removal of all Na^+ from the bathing solution⁵ (thereby blocking $\text{Na}^+/\text{Ca}^{2+}$ exchange) make measurement of Ca^{2+} -dependent light adaptation difficult. We re-examined *inaC* using criteria developed in *trp*²¹ to distinguish the fundamentally different mechanisms of adaptation and response inactivation. Furthermore, as the dissociated ommatidia preparation is unstable during the prolonged illumination regimes required to measure adaptation, we made measurements under strictly physiological conditions using intracellular recordings from intact flies. An obvious manifestation of adaptation is the rapid transition from peak to plateau following the onset of bright light. In *inaC* this phase is missing^{4,5}; instead, above a certain intensity, responses decay slowly to a low steady-state level (Fig. 1a, d), reaching baseline during intense white lights (data not shown). The classical, quantitative manifestation of light adaptation is a shift in the photoreceptor's operational range from dim to bright lights ($V/\log I$ curve)^{17,25,26}. As in *trp*^{21,27}, we found virtually no shift in *inaC* photoreceptors with dim or moderate backgrounds which clearly cause adaptation in wild-type (WT) photoreceptors (Fig. 1b, c). The parameters characterizing the $V/\log I$ curve σ (expressing adaptational shift), and V_{max} , are clearly different in wild type and *inaC* (Fig. 1c), indicating that the reduction in

§ To whom correspondence should be addressed.

FIG. 1 Adaptation is blocked in *inaC* while bright lights induce inactivation. **a**, Intracellular voltage responses to prolonged intense light in WT and *inaC* photoreceptors from intact flies (orange OG 570 edge filter, attenuated by log -1). In WT the peak response adapts rapidly to a pronounced maintained plateau. In *inaC* there is no rapid adaptation, but the receptor potential decays to a low steady baseline after 30 s. **b**, Response intensity functions ($V/\log I$) of the peak transient phase (see **a**) determined at different background intensities (OG 570 edge filter) in WT and *inaC*. Voltage increments above steady state are plotted. In WT all backgrounds induce a lateral shift in $V/\log I$ curve. In *inaC* a background of log -6 actually induces facilitation and there is no shift observed at log -5 . With a brighter ($>\log -4.0$) background there is mainly response compression. Data fitted (least-square deviations) with curves of the form: $V/V_{\max} = I^n/(I^n + \sigma^n)$ (ref. 26), where I is the light intensity, n is a constant (0.52 ± 0.16) and σ is the light intensity that elicits half of the maximal response ($V_{\max}$). **c**, The $V_{\max}$ values of the various curves in a family of $V/\log I$ curves determined at a range of backgrounds from log -6.0 to 0.0 , were normalized by the $V_{\max}$ of the dark adapted curve ($V_{\max}$) and plotted as a function of the corresponding σ values of the same curves, normalized by the σ of the dark adapted curve ($\bar{\sigma}$). Data from 6 *inaC* flies (triangles) and 8 wild-type flies (squares). The solid lines are regression curves which best fit the data points. The difference between WT and *inaC* points is highly statistically significant. Results similar to those of *inaC* were obtained from *trp* flies²⁷. The reduction in $V_{\max}$ in WT at medium and intense background lights is mainly due to the significant response to the background lights which limits the responding range in WT but not in *inaC* (see **a**). The long time required for measuring a family of $V/\log I$ curves makes it necessary to use incremental responses because the dark resting potential cannot be trusted in long recordings in intact *Drosophila*. **d**, Response intensity functions ($V/\log I$) of the steady-state phase measured in the experiments of **c** at various orange background lights >30 s after light onset. For WT, a typical curve was obtained. In *inaC*, significant responses were already observed with very dim backgrounds that elicited little or no response in WT. In *inaC* at higher background intensities the steady-state amplitude was reduced with increasing light intensities (OG 570 edge filter). The $V/\log I$ curves used in **c** at the highest orange background lights were obtained using white lights, where light of log -2.3 elicited response identical to log 0.0 of the orange light. When applying maximal white light as background, the response declined to zero level (not shown). Vertical bars are standard deviations of the mean ($n = 8$ for WT and $n = 9$ for *inaC*). **e**, Voltage responses to test flashes (60 J photographic strobe lamp with OG570 edge filter attenuated by log -1.5) delivered before and after a bright adapting orange light (150 W Xenon, 0.2-s pulse with OG570 edge filter). In WT the adapting stimulus shortens the response latency. In *inaC* although sensitivity is also reduced by the 'adapting' stimulus, latency is greatly prolonged. Vertical long arrows indicate stimulus onset.



METHODS. Flies were raised at 25°C; the wild-type strain was white-eyed Oregon R, the mutant allele was *inaC*^{P209} which is a null (protein-negative) allele of the eye-specific PKC⁵. In order to study stable light-adapted responses under physiological conditions we used standard intracellular recording techniques^{20,21,25}. Briefly, high-resistance (> 120 M Ω) intracellular pipettes were introduced into the retina of otherwise intact flies through a small hole cut in the cornea and sealed with high-vacuum silicon grease. Stimulation was with 150 W Xenon light source (XBO) with unattenuated intensity of the OG570 orange light of 42 mW per cm².

response amplitude in *inaC* is due to response compression (reduced $V_{\max}$) and not adaptation. As in *trp*, more intense backgrounds in *inaC* cause a collapse of steady-state potential, as might be expected if a limiting factor is depleted (Fig. 1a, d). Finally, light adaptation classically decreases response latency²⁸, whereas latency is increased following conditioning lights in *inaC* (Fig. 1e) and *trp*²¹. We conclude that light adaptation is severely reduced in *inaC* and suggest that, as in *trp*, the inactivation and response compression reflect exhaustion of the excitatory process.

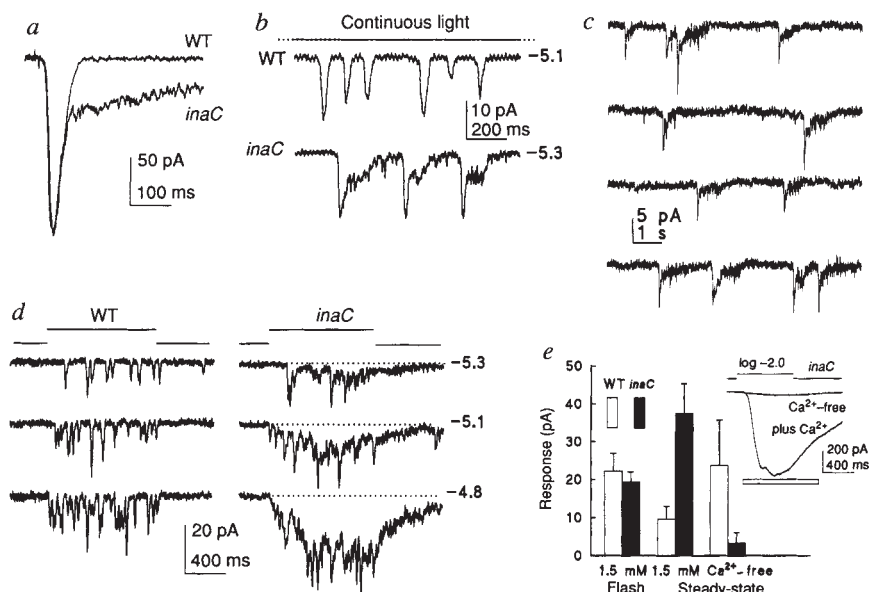
To analyse the underlying defect in *inaC*, we used whole-cell voltage clamp to isolate the light-induced current^{4,5,23,29}. Flash responses in *inaC* mutants show a characteristic two-phase decay. Although rising phase⁴, peak amplitude and initial decay are indistinguishable from wild type (Fig. 2a, e), as previously reported⁴, there is a very slow second component to the decay (Fig. 2a). We show here that a similar behaviour can be seen at the level of the single quantum bumps. In wild-type photoreceptors, quantum bumps occur as discrete events of ~ 10 pA and 30 ms duration. In *inaC*, the bumps show similar amplitude and rise time, but the decay again shows two distinct phases, one being very slow and displaying high frequency noise suggestive of random channel activity (Fig. 2b, c). With increasing intensities of continuous dim light, wild-type responses reach a noisy

steady-state within a few tens of milliseconds (Fig. 2d). In *inaC*, however, responses continue rising along a ramp-like trajectory for more than one second, reaching amplitudes on average four times greater than in wild type (Figs 2d, e and 1d). This is due to the summation of the slowly decaying component from successive bumps, which is revealed at light off. Significantly, this facilitation of the dim light steady-state response in *inaC* is reversed if recordings are made after several minutes exposure to Ca^{2+} -free Ringer's solution. Sensitivity in *inaC* is now greatly reduced but if extracellular Ca^{2+} is transiently raised, the response is rapidly restored (Fig. 2e), which is exactly the opposite of what would have been predicted if *inaC* were subject to excess Ca^{2+} -mediated adaptation.

As in other rhabdomeric photoreceptors^{30,31}, recent evidence indicates that there may be at least two light-activated conductances in *Drosophila* photoreceptors, one highly selective for Ca^{2+} and absent in the *trp* mutant^{23,24,34}. To test whether the activity of both conductances requires PKC for their regulation, we constructed a double mutant (*inaC;trp*) lacking both PKC and the *trp*-dependent channels. Just as in the WT/*inaC* comparison (Fig. 2a), when comparing *inaC;trp* with *trp*, the rising phases of flash responses overlap, whereas the response decay in the double mutant is prolonged (Fig. 3a, b). The non-*trp* channels in both *trp* and *inaC;trp* are characterized by a

FIG. 2 Whole-cell current responses in *inaC* and wild-type photoreceptors. *a*, Responses to 10-ms flashes (relative intensity, log -2.5) in adult *inaC* and WT. Initially responses overlap, but the *inaC* response has a characteristic slowly decaying tail with high-frequency noise. *b*, Quantum bumps recorded in response to continuous dim illumination (p15 pupae). In *inaC* although bump rise and initial decay appear similar to wild type, there is again a conspicuous tail. *c*, Continuous record of *inaC* bumps recorded on a slower time base (continuous illumination, log -6.0, adult fly). *d*, In WT photoreceptor (adult), steps of light evoke discrete quantum bumps. These sum to produce a noisy steady-state response (reached in less than 100 ms) as the intensity is increased (log *I* indicated). In *inaC*, summation of the incompletely terminated bumps results in a slowly rising ramp of current. *e*, With normal (1.5 mM) extracellular Ca^{2+} , the peak response amplitudes of *inaC* photoreceptors (solid bars) to short flashes (10ms, log -3.5) are indistinguishable from WT (left), but the steady-state reached during prolonged illumination (log -4.5) is 4–5 times greater (centre). After 10–40 min exposure to Ca^{2+} -free Ringer's solution, steady-state responses in *inaC* are greatly reduced compared to WT (right). Means $\pm$ s.d. from between 5 and 13 cells (2 or 3 flies). Each pairwise comparison was performed under identical conditions in adult flies. Inset: Whole-cell recording from an adult *inaC* photoreceptor bathed in nominally zero-calcium Ringer's solution. Compared to WT, the control response to a light pulse is greatly reduced (see bar graph). A second response (plus Ca^{2+} , elicited 5 s later) is massively facilitated when normal- Ca^{2+} Ringer's (1.6 mM) was directed onto the cell using a picrospritizer during the time indicated by the bar. Representative of results in 5 cells.

METHODS. Whole-cell recordings were made from dissociated ommatidia as previously described^{23,29}. To compare responses in absolute terms, we used newly enclosed (<4h) adults as there is a continuous increase in sensitivity during pupal life (otherwise there were no relevant differences between adult and pupal photoreceptors). The pipette solution contained (in mM): 135 K-gluconate, 2



MgSO_4 , 10 TES, 0.1 EGTA, and no added Ca^{2+} (pH 7.15); 4 Mg-ATP and 0.4 GTP were also included in some experiments. Bath Ringer contained (in mM) 120 NaCl, 5 KCl, 10 TES, 25 sucrose, 5 proline and 8 MgSO_4 (pH 7.15); CaCl_2 1.5 mM unless otherwise stated. Stimulation was with a QI light filtered by a OG530 filter and Wratten (Kodak) ND filters, directed onto the preparation by a light guide positioned 2 cm over the bath. Recordings were made using an Axopatch 1-B amplifier using pClamp software (Axon Inc). Series resistances in the range 10–20 M Ω , clamp time constant less than 1 ms. Holding potential: *a* and *e*, recorded at -60mV; *b–d* recorded at -80mV and with low (0.5 mM) Ca_o to accentuate the bumps. Inset: Whole-cell recordings; methods and solutions as in *a–d*; bath Ringer contained no added Ca^{2+} (free $\text{Ca}^{2+} \sim 2 \mu\text{M}$, Mg^{2+} 8 mM). A similar Ringer but with 1.6 mM CaCl_2 and no MgSO_4 (calculated to sustain an identical current through the light-sensitive channels²³) was pressure-ejected from a picrospritizer pipette (tip diameter, 2 μm), positioned $\sim 20 \mu\text{m}$ from the recorded cell.

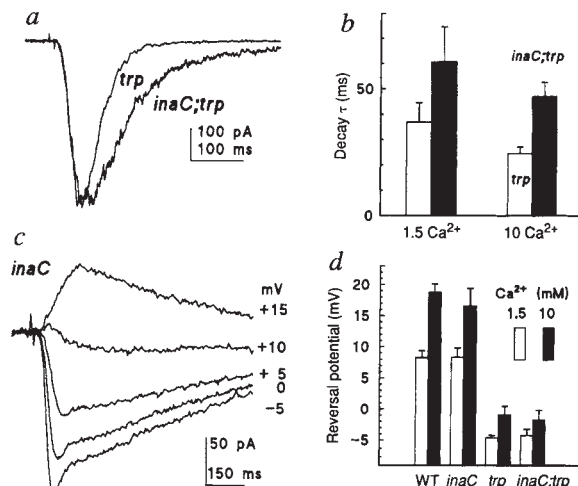
negative reversal potential which shows little Ca^{2+} dependency; however, in *inaC* the reversal potential of even the slowly decaying tail of the response is similar to wild type and strongly Ca^{2+} -dependent, indicating that here the *trp*-dependent channels are also active (Fig. 3c, d). These results indicate that both

suspected channel classes require PKC for their activity to be terminated normally.

PKC probably has multiple substrates, but by what mechanism might it act to terminate quantum bumps? The results shown in Fig. 3 suggest action at a level required for activation of both

FIG. 3 The *inaC* mutation affects both *trp*- and non-*trp*-dependent channels. *a*, Responses to 10-ms flashes (log -1.5) recorded under identical condition in adult *trp* and *inaC;trp*. Bath contained normal Ringer (1.5 mM Ca^{2+}). *b*, Averaged decay time constants (single exponential fits to the decay phase of the flash responses) in *trp* (open bars) and *inaC;trp* (solid bars) in both 1.5 mM and 10 mM Ca^{2+} Ringer's solution. Means $\pm$ s.d. from 11–19 cells (3–4 flies) in each case. The difference between *trp* and *inaC;trp* decay times is highly significant ($P < 0.001$) at both Ca^{2+} concentrations. *c*, Flash responses (10 ms, log -2.5) in *inaC* recorded at different holding potentials in 1.5 mM Ca^{2+} Ringer. The tails of the responses reverse near +10 mV indicative of *trp*-dependent channels²³. Notice also the marked voltage dependence of the initial phase of response termination and the biphasic response around +10mV, which we attribute to early activation of non-*trp*-dependent channels²³. *d*, Reversal potentials of WT, *inaC*, *trp* and *inaC;trp*; measured in 1.5 mM and 10 mM Ca^{2+} Ringer. Values in *inaC* are similar to WT, whereas both *trp* and *inaC;trp* have more negative reversal potentials with less dependency on external Ca^{2+} (ref. 23). Means $\pm$ s.d. from between 4 and 8 cells (2–3 flies) in each case.

METHODS. The *inaC;trp* double mutant was generated over a 5-generation cross using stocks of *inaC*^{p209} and *trp*^{CM} after first crossing with flies with balancers on the appropriate chromosomes. Reversal potential measurements were made with an electrode solution con-



taining (in mM) 120 CsCl, 15 TEA-Cl, 10 TES, 2 MgSO_4 to block K channel activity²⁹ and have been corrected for a -3mV junction potential.

trp- and non-*trp*-dependent channels. One potential substrate⁵ is phospholipase C (PLC) which generates InsP_3 . But as application of InsP_3 itself induces bump-like events^{7,9,12} and as a temperature-sensitive mutation of PLC affects bump latency dispersion, but not bump shape³², bumps are probably initiated downstream from PLC, suggesting that the defect in *inaC* also occurs at a later stage. InsP_3 causes release of Ca^{2+} from intracellular stores^{2,12,33}; if quantal release from these stores is responsible for bump formation, then a failure to terminate the resulting rise in cytosolic Ca^{2+} could account for the characteristic bump shape observed in *inaC*. Candidate processes include negative feedback at the release channel (InsP_3 receptor)¹² or activation of a sequestering mechanism (Ca^{2+} -ATPase). In addition, such an explanation can account for both the facilitation seen during dim backgrounds (Fig. 2d), and also for the inactivation by intense backgrounds (Fig. 1a, d) or by exposure to Ca^{2+} -free Ringer's solution (Fig. 2e). This seemingly paradoxical behaviour can be reconciled by assuming that the amount of Ca^{2+} released from the stores determines the level of excitation: facilitation at low light levels would represent greater net Ca^{2+} release, whereas at higher intensities, or in the absence of extracellular Ca^{2+} , failure to terminate release efficiently could lead to depletion of the stores and collapse of the response.

Finally, although we have shown that PKC is required for light adaptation, we do not exclude the possibility of other Ca^{2+} -mediated negative feedback mechanisms. An example is the initial phase of bump termination (Fig. 2b), which even in *inaC* accelerates as extracellular Ca^{2+} is raised (data not shown) or as the cell is hyperpolarized (Fig. 3c).

In summary, we have determined the defect in *inaC* to be the failure of individual quantum bumps to terminate (Fig. 2b, c) and have shown that under physiological conditions light adaptation is severely reduced (Fig. 1b, c). Furthermore, on the assumption that Ca^{2+} is required for excitation, our hypothesis that PKC is required to terminate the light-induced rise in cytosolic Ca^{2+} readily and economically explains the diverse pleiotropic consequences of the *inaC* mutation, including

response-inactivation by intense light, facilitation to dim lights and slow termination of the light response. □

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1. Nishizuka, Y. *Nature* **334**, 661–665 (1988).
2. Berridge, M. J. A. *Rev. Biochem.* **56**, 159–193 (1987).
3. Schaeffer, E., Smith, D., Mardon, G., Quinn, W. & Zuker, C. S. *Cell* **57**, 403–412 (1989).
4. Ranganathan, R., Harris, G. L., Stevens, C. F. & Zuker, C. S. *Nature* **354**, 230–232 (1991).
5. Smith, D. P. *et al.* *Science* **254**, 1478–1484 (1991).
6. Pak, W. L. in *Neurogenetics, Genetic Approach to the Nervous System* (ed. Breakfield, X.) 67–99 (Elsevier, New York, 1979).
7. Fein, A., Payne, R., Corson, D. W., Berridge, M. J. & Irvine, R. F. *Nature* **311**, 157–160 (1984).
8. Brown, J. E. *et al.* *Nature* **311**, 160–163 (1984).
9. Devary, O. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **84**, 6939–6943 (1987).
10. Bloomquist, B. T. *et al.* *Cell* **54**, 723–733 (1988).
11. Minke, B. & Selinger, Z. in *Progress in Retinal Research* **11** (eds Osborne, N. N. & Chader, G. J.) 99–124 (Pergamon, Oxford, 1991).
12. Payne, R., Walz, B., Levy, S. & Fein, A. *Phil. Trans. R. Soc. B* **320**, 359–379 (1988).
13. Yeandle, S. *Am. J. Ophthalmol.* **46**, 82–87 (1958).
14. Wong, F. *Nature* **276**, 76–79 (1978).
15. Stieve, H. in *The Molecular Mechanism of Photoreception* (ed. Stieve, H.) 199–230 (Springer, New York, 1986).
16. Dodge, F. A. Jr, Knight, B. W. & Toyoda, J. *Science* **160**, 88–90 (1968).
17. Shapley, R. & Enroth-Cugell, C. in *Progress in Retinal Research* vol. 3 (eds Osborne, N. N. & Chader, G. J.) 263–346 (Pergamon, Oxford, 1984).
18. Brown, J. E. & Blinks, J. R. *J. gen. Physiol.* **64**, 643–665 (1974).
19. Minke, B. & Payne, R. *J. Neurosci.* **11**, 900–909 (1991).
20. Minke, B., Wu, C.-F. & Pak, W. L. *Nature* **258**, 84–87 (1975).
21. Minke, B. *J. gen. Physiol.* **79**, 361–385 (1982).
22. Howard, J. J. *exp. Biol.* **113**, 471–475 (1984).
23. Hardie, R. C. & Minke, B. *Neuron* **8**, 643–651 (1992).
24. Phillips, A. M., Bull, A. & Kelly, L. E. *Neuron* **8**, 631–642 (1992).
25. Laughlin, S. B. & Hardie, R. C. *J. comp. Physiol.* **128**, 319–340 (1978).
26. Kleinschmidt, J. & Dowling, E. J. *gen. Physiol.* **66**, 617–648 (1975).
27. Minke, B. & Armon, E. *Photochem. Photobiol.* **32**, 553–562 (1986).
28. Brown, J. E. & Lisman, J. E. *Nature* **258**, 252–254 (1975).
29. Hardie, R. C. *Proc. R. Soc. Lond. B* **245**, 203–210 (1991).
30. Nasi, E. *J. gen. Physiol.* **97**, 55–72 (1991).
31. Nagy, K. Q. *Rev. Biophys.* **24**, 165–226 (1991).
32. Pak, W. L., Ostroy, S. E., Deland, M. C. & Wu, C.-F. *Science* **194**, 956–959 (1976).
33. Baumann, O. & Walz, B. *J. comp. Physiol. A* **165**, 627–636 (1989).
34. Montell, C. & Rubin, G. M. *Neuron* **2**, 1313–1323 (1989).

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A conserved mitotic kinase active at late anaphase–telophase in syncytial *Drosophila* embryos

Brian Fenton* & David M. Glover

Cancer Research Campaign Cell Cycle Genetics Group, Department of Anatomy and Physiology, Medical Sciences Institute, University of Dundee, Dundee DD14HN, UK

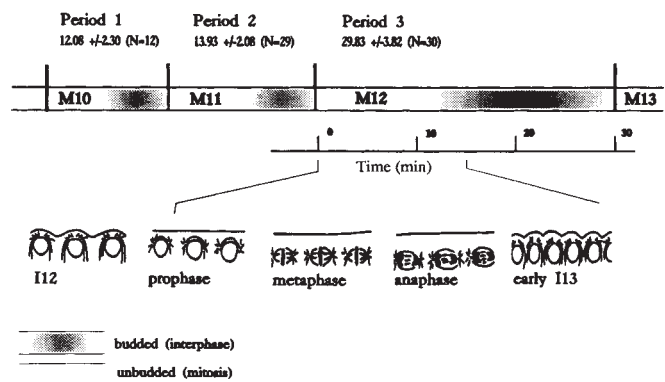
MUTATIONS in the *Drosophila* gene *polo* cause abnormal mitotic and meiotic divisions^{1,2}. This gene encodes a 577-amino-acid protein that has an N-terminal putative kinase domain and a 300-residue C-terminal domain². In budding yeast, a homologous kinase is encoded by *CDC5* (ref. 3), a gene required for nuclear division late in the mitotic cycle⁴ and during meiosis⁵. Murine homologues have also been described^{6,7}. Here we show that the *polo* gene product immunoprecipitated from extracts of single *Drosophila* embryos can phosphorylate casein *in vitro*, and that the kinase activity peaks cyclically at late anaphase/telophase. This contrasts with the cyclical activity of cyclin B-associated p34^{cdc2} kinase, which is maximal upon entry into

mitosis during the rapid cycles of mitosis in the syncytium.

Measurements of kinase activity in relation to the mitotic cycle have not yet been described in *Drosophila* owing to lack of an efficient protocol for synchronizing cultured *Drosophila* cells and an inability to achieve synchronous development of populations of embryos. But as the first 13 mitoses of embryonic development take place in a para-synchronous fashion in a shared cytoplasm⁸, assays carried out on extracts made from single embryos should sample a very narrow time-window in mitosis, and thus provide a powerful system for analysing enzyme activities at specific mitotic stages. Nuclei are located in the interior of the embryo for the initial divisions, but migrate towards the cortex during cycles 8 and 9. Bud structures over the nuclei during interphase that break down during mitosis are visible by light microscopy for the next four division cycles, which become progressively longer (Fig. 1)⁸. We chose to assay extracts made from single embryos for kinase activity as they progress through mitosis 12 and into the interphase of cycle 13 (period 3, Fig. 1). To increase further the resolution of our sampling, we maintained the embryos at 18°C to slow the division cycle (Fig. 1). To examine the temporal progression through mitosis, we selected individual embryos for fixation and immunostaining at specific times following what we take to be disappearance of buds at the onset of mitosis 12 (Fig. 2). Nuclei are well into prophase at outset of this time course, showing condensed chromosomes, and fully separated centrosomes. The reformation of cortical buds corresponds to late anaphase–telophase (8–10 min; Fig. 2).

* Present address: Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK.

FIG. 1 Timing of the budding cycles at 18°C. Approximately 100 dechorionated embryos were placed in a 5-cm Petri dish to which were added 2 ml immunoprecipitation buffer (IP buffer: 50mM Tris, pH 8.0, 1mM EDTA, 0.5% Triton-X100 and 1 $\mu\text{g ml}^{-1}$ each of aprotinin, leupeptin and pepstatin A). Individual syncytial embryos, that had yet to form pole cells, were sucked up along with 10 μl immunoprecipitation buffer and placed into wells of a microtitre plate. The budding cycles of individual embryos were followed on an inverted microscope in a room maintained at 18°C. Three time periods were measured, corresponding to mitosis 10 and interphase 11 (period 1), mitosis 11 and interphase 12 (period 2) and mitosis 12 and interphase 13 (period 3). Immunostaining (Fig. 2) indicated that the point which we define as the onset of bud breakdown is into prophase, and that the reappearance of buds corresponds to late anaphase. These unbudded phases lasted 4.42 (± 0.49), 5.33 (± 1.68) and 6.98 (± 1.66) min for mitoses 10, 11 and 12 respectively. Single embryos were picked for subsequent experiments at points along the indicated time-line from the onset of period 3.



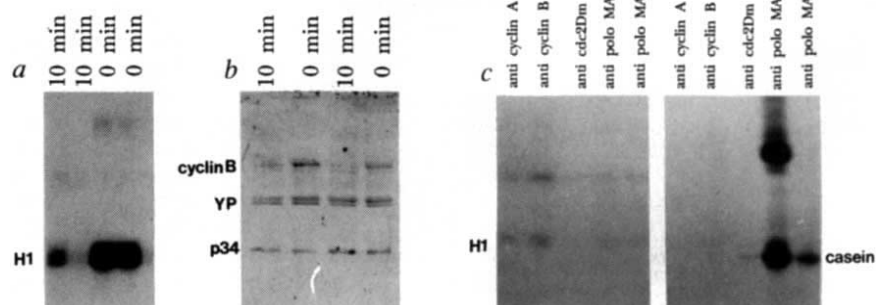
We investigated the appearance of polo kinase activity relative to $p34^{cdc2}$ kinase during progression through mitosis. The histone H1 kinase activity associated with a cyclin B immunoprecipitate of a single embryo extract is high at the time when cortical buds disappear (0 min) and low when they are reappearing (10 min; Fig. 3a), consistent with the expected time of maximal activity of $p34^{cdc2}$ kinase upon entry into mitosis. Immunoblotting of such extracts indicated comparable levels of protein of M_r 34,000 (34K) detected by a monoclonal antibody directed against the conserved 'PSTAIRES' region of $p34^{cdc2}$ (ref. 9). There are significantly higher levels of cyclin B at 0 min compared to 10 min, suggesting that some breakdown of cyclin B occurs at the metaphase-anaphase transition (Fig. 3b). As earlier studies have shown that neither cyclin A nor B is completely broken down during anaphase¹⁰, the present finding suggests that localized degradation of cyclin B must occur. This could in part regulate the activity of $p34^{cdc2}$ kinase and explain the pattern of activity that we recover. However, higher-resolution blots (not shown) indicate the existence of a second form of $p34^{cdc2}$ at 0 min, suggesting that phosphorylation may also positively regulate the enzyme in the syncytial cycles. Before determining the times at which polo kinase was active, we first looked for suitable substrates to differentiate its activity

from $p34^{cdc2}$ kinase in extracts of a small number of non-staged embryos expected to be representative of all cell-cycle stages. We found that immunoprecipitates formed using anti-cyclin B antibodies would preferentially phosphorylate histone H1 rather than casein, whereas immunoprecipitates formed using anti-polo antibodies have the opposite specificity (Fig. 3c).

The results of the analysis of single embryo extracts for both cyclin B-associated H1 kinase and polo kinase in two experiments examining progression through mitosis 12 and into cycle 13 are given in Fig. 4. The kinase activity associated with cyclin B is maximal in embryos entering mitosis (mitosis 12, Fig. 4a; and mitosis 13, Fig. 4c). The metaphase-anaphase transition occurs at around 5–8 min (Fig. 2), at which time the H1 kinase activity has fallen to its lowest level. Polo kinase, on the other hand, shows a peak of activity at 10–12 min (Fig. 4b, d), corresponding to late anaphase-telophase (Fig. 2). (The variation observed here reflects both the errors inherent in timing cortical bud breakdown and the natural variation in length of the mitotic cycle from one embryo to another; see Fig. 1 legend).

The polo kinase is highly conserved. Its homologues from budding yeast, *CDC5* (ref. 3), and mouse, Plk (Polo-like kinase)⁶ and Snk (serum-induced kinase)⁷ show respective identities of 52, 65 and 52% in the kinase domain, and 8, 43 and

FIG. 3 Detection of cyclin B associated H1 kinase and immunoprecipitable polo kinase. a, Individual embryos were selected at cortical bud breakdown (0 min), or following their re-formation (10 min). They were disrupted and homogenized with a sterile needle, and each homogenate removed and placed in a 1.5 ml Eppendorf tube which was frozen on dry ice. This process took <30 s. Tubes were thawed, diluted to 100 μl with IP buffer, pre-cleared with 10 μl protein G in IP buffer and immunoprecipitated with antibodies to cyclin B precoupled to protein-G beads. Precipitates were then washed once in IP buffer containing 500mM NaCl and twice in IP buffer, and assayed for histone H1 kinase activity. Buffers and conditions used were as described¹⁷, except that the total reaction volume was reduced to 15 μl . Phosphorylated substrates were detected by SDS-PAGE autoradiography. b, Extracts from individual embryos selected at 0 min or 10 min following cortical bud breakdown were subjected to SDS-PAGE and sequential immunoblotting with an anti-PSTAIRES monoclonal antibody⁹ to reveal $p34$, and anti-cyclin B antiserum¹⁶. The antibodies used in this experiment also give nonspecific staining of the highly abundant yolk proteins (YP). Note that the levels of yolk proteins and $p34$ remain constant, whereas cyclin B is more abundant in extracts of embryos whose cortical buds are breaking down. c, A collection of about 50



syncytial embryos at random stages in the division cycle was homogenized and precipitated with either anti-cyclin A (Rb270 (ref. 16)), anti-cyclin B (Rb271 (ref. 16)), anti-cdc2Dm monoclonal antibody (Alphay and D.M.G., unpublished), or anti-polo kinase monoclonal antibodies (MA81 and MA75 (ref. 2)). Immunoprecipitates were divided into two aliquots and assayed for their ability to phosphorylate histone H1 (left panel), or casein (right panel). The immunoprecipitate produced by the anti-cdc2 antibody C845 did not give any kinase activity significantly above background, whereas anti-cyclin A or B and anti-Polo precipitates are preferentially active against histone H1 and casein, respectively.

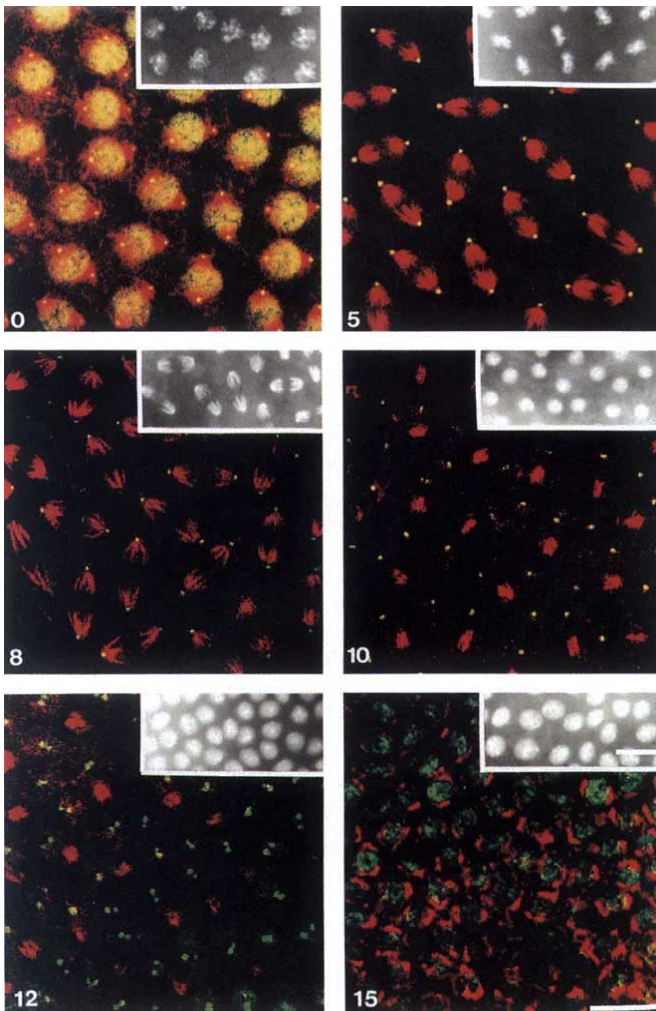


FIG. 2 Progression of the 12th mitotic cycle in relation to cortical budding at 18°C. Embryos, picked at 0, 5, 8, 10, 12 and 15 min after the start of period 3 (Fig. 1), were removed and fixed directly in 1:1 37% formaldehyde : heptane and then processed as described¹³. Embryos were then stained with Hoechst, Rb188 (yellow-green stain) or YL1/2 (anti-tubulin) (red stain). The Rb188 antibody¹⁴ recognizes the Bx63 nuclear antigen that associates with centrosomes during mitosis^{14,15}. Microtubule and Bx63 staining patterns were detected by confocal microscopy. Hoechst staining of DNA was observed by conventional fluorescence microscopy (insets). Four embryos examined at each time-point were found to show no significant variation. At time 0 the nuclei contain condensing prophase chromosomes; the centrosomes are on opposing sides of the nucleus, and spindles are forming. Some Bx63 antigen is still associated with the nucleus. At 5 min, spindles have formed and chromosomes have congressed onto the metaphase plate. Bx63 antigen is found exclusively at the centrosome. At 8 min, anaphase is nearly complete. At 10 min, the chromosomes have decondensed and the spindles disappeared, leaving a residual midbody (red). Duplicated centrosomes are beginning to separate, but the Bx63 antigen is still centrosomal. At 12 min all of the duplicated centrosomes are well separated. The midbodies are disappearing and the Bx63 antigen is returning to the nucleus. By 15 min the interphase pattern of staining has returned with the Bx63 antigen being totally nuclear. Microtubule networks now surround the nuclei which have started to increase in size. Scale bars, 10 μ m.

35% in the C-terminal domain, the latter homologies lying in three distinct blocks^{3,6,7}. Moreover, a human Plk has been isolated with comparable identity to the *Drosophila* gene as murine Plk (R. Golsteyn and E. Nigg, personal communication), and we have identified homologous genes from both fission yeast and *Xenopus* (H. Ohkura and D. M. G., unpublished). The murine genes are expressed only in proliferating cells, but whether they have a role in mitosis has not yet been established. In mutants of the budding yeast homologue, *CDC5*, nuclear division is arrested at a late stage and the spindle is elongated¹¹. Furthermore, when temperature-sensitive *cdc5* mutants are shifted to restrictive temperature after initiating the first meiotic division, two spindles form that do not elongate, and the four poles encapsulate into two diploid spores⁵. Although the activity of *CDC5* kinase has not been determined with respect to cell-cycle progression, its transcript is periodically accumulated around G2/M (ref. 3). The *polo* mutation results in a high mitotic index in larval neuroblasts with a wide range of defects, including monopolar spindles and spindles with broad poles^{1,2}. Multipolar spindles and non-disjunction are seen in male meiosis¹. This mutation is leaky and does not lead to arrest at a distinct mitotic stage, the phenotype only being seen as maternally supplied protein becomes limiting. Nevertheless, there are considerable similarities between the phenotypes of

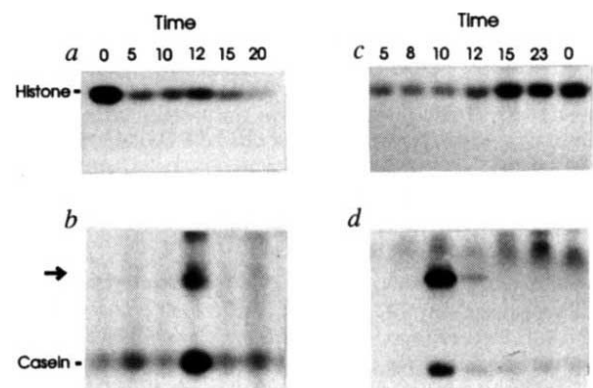


FIG. 4 Time course of cyclin B-associated HI kinase and polo kinase activity. Individual embryos were picked at the times in minutes indicated above each lane, representing progression through period 3 (Fig. 1). Results are shown from two experiments. In the experiment shown in the right-hand panel, the last time point represents entry into mitosis 13. Individual embryos were prepared for immunoprecipitation as described in Fig. 3 legend. To maximize the use of material, as well as provide internal controls, each homogenate was immunoprecipitated first with anti-cyclin-B, the precipitate being assayed for the ability to phosphorylate histone H1 (a and c), and the supernatant then subjected to immunoprecipitation using anti-polo antibodies and assayed for casein kinase activity (b and d). The kinase activity associated with cyclin B is high at the start of mitosis 12 and 13 (tracks 0 in a and c, respectively), a time point shown to be prophase in Fig. 2. This activity may also be increasing towards the end of the interphase periods, although we have observed some variation in the duration of the interphase period from one embryo to another, thus making it difficult to be sure of the precise interphase state (tracks 15 and 23 in c). This is likely to account for the variation of HI kinase activity observed between the two experiments. The casein kinase activity associated with polo kinase is high at times of 10 to 12 min after the start of period 3 (track 12 in b, and tracks 10 and 12 in d). The upper band (indicated by an arrow) in the casein kinase assays appears to be due to auto-phosphorylation of Polo protein. We have also detected Polo kinase activity at a comparable stage of mitosis 13 (results not shown).

mutations in the homologous genes of the two organisms, and we are currently testing whether genes from budding yeast and *Drosophila* are functionally homologous. A number of circumstantial observations suggest that *polo/CDC5* may regulate microtubule behaviour. *cdc5* mutants show an unusual interaction with MBC, a drug that binds tubulin and depolymerizes microtubules¹²; and there is a strong interaction of *polo* with mutations in *asp* which appear to affect microtubule stability (C. Gonzalez, C. E. Sunkel and D.M.G., unpublished). The mitotic stage at which Polo kinase appears to be maximally active would be consistent with a role in orchestrating the changes in microtubule organization that have to occur late in anaphase and in telophase. The availability of mutations in the genes and antibodies to their proteins now offers the means of exploring some of these potential roles for the enzyme. □

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1. Sunkel, C. E. & Glover, D. M. *J. Cell Sci.* **89**, 25–38 (1988).
2. Llamazares, S. *et al. Genes Dev.* **5**, 2153–2165 (1991).

3. Kitada, K., Johnston, L. H. & Sugino, A. *Molec. cell. Biol.* (in the press).
4. Hartwell, L. H. *J. Bact.* **115**, 966–974 (1973).
5. Schild, D. & Byers, B. *Genetics* **96**, 859–876 (1980).
6. Clay, F. & Dunn, A. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
7. Simmons, D. L., Neel, B. G., Stevens, R., Evett, G. & Erikson, R. *Molec. cell. Biol.* **12**, 4164–4169 (1992).
8. Foe, V. & Alberts, B. *J. Cell. Sci.* **61**, 31–70 (1983).
9. Yamashita, M., Yoshikuni, M., Hirai, T., Fukada, S. & Nagahama, Y. *Dev. Growth Differ.* **33**, 617–624 (1991).
10. Maldonado-Codina, G. & Glover, D. M. *J. Cell Biol.* **116**, 967–976 (1992).
11. Byers, B. & Goetsch, L. *Cold Spring Harbor Symp. quant. Biol.* **38**, 123–131 (1974).
12. Wood, J. S. & Hartwell, L. H. *J. Cell Biol.* **94**, 718–726 (1982).
13. Gonzalez, C. & Glover, D. M. in *The Cell Cycle: A Practical Approach* (eds Brooks, R. & Fantes, P.) (IRL, Oxford, 1993).
14. Whitfield, W., Millar, S. E., Saumweber, H., Frasch, M. & Glover, D. M. *J. Cell Sci.* **89**, 467–480 (1988).
15. Frasch, M., Glover, D. M. & Saumweber, H. *J. Cell Sci.* **82**, 155–172 (1985).
16. Whitfield, W., Gonzalez, C., Maldonado-Codina, G. & Glover, D. M. *EMBO J.* **9**, 2563–2572 (1990).
17. Marshak, D. R., Vandenbergh, M. T., Seuk-Bae, Y. & Je Yu, I. *J. Cell. Biochem.* **45**, 391–400 (1991).

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Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma

Anne Crozat*, Pierre Åman†, Nils Mandahl† & David Ron*‡

* Departments of Medicine, Cell Biology and the Kaplan Cancer Center, NYU Medical Center, New York, New York 10016, USA

† Department of Clinical Genetics, University Hospital, Lund S-22185, Sweden

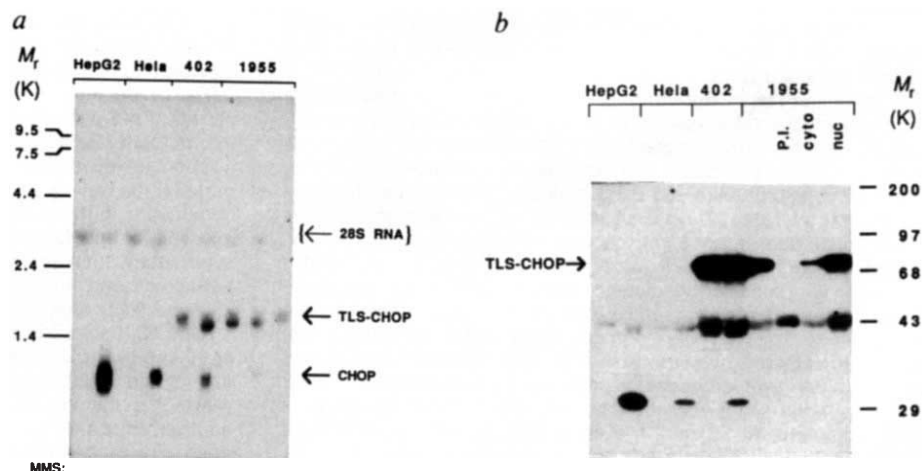
HUMAN myxoid liposarcomas contain a characteristic chromosomal translocation, t(12;16)(q13;p11)^{1,2}, that is associated with a structural rearrangement of the gene encoding CHOP³, a growth arrest and DNA-damage inducible member of the C/EBP family of transcription factors^{4,5} residing on 12q13.1⁶. Using a CHOP-specific complementary probe and antiserum we report

here the presence of an abnormal CHOP transcript and protein in these tumours. Cloning of the translocation-associated *CHOP* gene product revealed a fusion between *CHOP* and a gene provisionally named *TLS* (translocated in liposarcoma). *TLS* is a novel nuclear RNA-binding protein with extensive sequence similarity to EWS⁷, the product of a gene commonly translocated in Ewing's sarcoma. In *TLS-CHOP* the RNA-binding domain of *TLS* is replaced by the DNA-binding and leucine zipper dimerization domain of *CHOP*. Targeting of a conserved effector domain of RNA-binding proteins to DNA may play a role in tumour formation.

Northern blot analysis of RNA from myxoid liposarcoma (MLPS) tumour cells containing the t(12;16) rearrangement demonstrated the constitutive expression of an abnormally large *CHOP* transcript (Fig. 1a, lanes 5–9). A normal sized *CHOP* messenger RNA could also be induced in these cells by treatment with a DNA-damaging agent (lanes 6 and 8). Tumour cells that do not contain the t(12;16) rearrangement express only the normal sized *CHOP* transcript (lanes 2 and 4). To determine if the abnormal *CHOP* transcript was associated with the production of an abnormal protein, we immunoprecipitated the

FIG. 1 Myxoid liposarcoma cells contain an abnormal *CHOP* transcript and protein. *a*, Northern blot RNA from hepatoblastoma (HepG2), cervical carcinoma (HeLa) and myxoid liposarcoma (402/91 and 1955/91) cell lines, hybridized at high stringency with a *CHOP* probe. Where indicated, the normal *CHOP* gene was induced by treatment of the cultured cells with the DNA-damaging agent methylmethanesulphonate (MMS). Position of the normal *CHOP* transcript and abnormal *TLS-CHOP* transcript is indicated, as are the 28S ribosomal bands that react weakly with the *CHOP* probe. Lanes 1–8 contain total cellular RNA and lane 9 the poly(A)⁺ fraction. Longer exposure shows the induction of *CHOP* by MMS also in the 1955/91 cell line. *b*, C/EBPβ 'zipper blot' of proteins immune precipitated from cellular extracts with a *CHOP*-specific antiserum. Pre-immune serum was used as control in lane 8. Whole-cell extracts were used in lanes 1–8, lane 9 contains cytosol and lane 10 nuclear extract. The position of normal (*CHOP*) and abnormal (*TLS-CHOP*) proteins is indicated, as are the molecular mass markers. The asterisks to the left of the autoradiogram marks the superposition of a nonspecific zipper-reactive protein and proteins that specifically coprecipitate with *TLS-CHOP* in the myxoid liposarcoma cells.

METHODS. *a*, Freshly fed cultured cells (1955/91 and 402/91) are MLPS cell lines established by immortalizing cells from two different tumours with SV40



large-T antigen) were treated for 4 h with MMS (100 μg ml⁻¹). RNA preparation and northern blot hybridization with the *CHOP* probe were as previously described³. *b*, Cellular proteins were precipitated with a *CHOP*-specific antibody, resolved by 10% SDS-PAGE, transferred to a nitrocellulose filter and the filter was reacted with a 'zipper-probe' consisting of the bacterially expressed ³²P-labelled dimerization domain of C/EBPβ⁵.

TLS-CHOP

a

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1  ATGCTCAGTCTCCAGGCGTGGTCTCAGCGGTGTTGGAACCTCGTTCCTGCTTGCCT
61  GTGCGCGCGTCCGCGGACATGGCTCAAAACGATTATACCAACCAAGCAACCAAGCTAT
    M A S N D Y T Q Q A T Q S Y
121  GGGGCTACCCACCCAGCGCGGCGAGGCTATCCAGCAGAGCAGTCCAGCCTACGGA
15  G A Y P T Q P G Q G Y S Q Q S S Q P Y G
181  CAGCAGAGTTACAGTGGTTATAGCCAGTCCAGGACACTTCAGGCTATGGCCAGAGCAGC
35  Q Q S Y S G Y S Q S T D T S G Y G Q S S
241  TATTCCTTTATGGCCAGAGCCAGAACACAGGCTATGGAACCTCAGTCAACTCCCAGGGA
55  Y S S Y G Q S Q N T G Y G T Q S T P Q G
301  TATGGCTCGACTGGCGGCTATGGCAGTAGCCAGAGCTCCCAATCGTCTACGGGACGAG
75  Y G S T G G Y S Q S S Q S S Q S Y G Q Q
361  TCCTCCTACCTGGTATGGCCAGCAGCAGCTCCAGCAGCAGCTCGGGAAGTTACGGT
95  S S Y P G Y G Q Q P A P S S T S G S Y G
421  AGCAGTTCTCAGAGCAGCAGTATGGCAGCCAGAGTGGGAGCTACAGCCAGCAGCCT
115  S S S Q S S S G Y S Q P Q S S Y S Y S G Q Q
481  AGCTATGGTGGACAGCAGAAAGCTATGGACAGCAGAAAGCTATAATCCCCCTCAGGCG
135  S Y G G Q Q Q S Y G Q Q Q S Y N P P Q G
541  TATGGACAGCAGAACCAATACACAGCAGCAGTGGTGGAGGTGGAGGTGGAGGTGGA
155  Y G Q Q N Q N Q S Y S G G G G G G G G G G G
601  GGTAATATGGCCAAAGTCAATCTCCATGAGTAGTGGTGGTGGCAGTGGTGGCGTTAT
175  G N Y G Q D Q S S M S S G G G S G G G Y
661  GGCAATCAAGACAGAGTGGTGGAGGTGGCAGCGGTGGCTATGACAGCAGGACCGTGA
195  G N Q D Q S S G S S G Y G Q Q Q S Y G
721  GGCGCGGCGAGGGTGGCAGTGGTGGCGCGCGCGCGCGCGGTGGTGGTTACAACCGC
215  G R G R G G S G G G G G G G G G G G G Y N R
781  AGCAGTGGTGGCTATGAACCCAGAGGTCGTGGAGGTGGCGGTGGAGGACAGAGTGGCATG
235  S S G G Y E P R G G G R G G R G G R G G
841  GGCGGAAGTACCGTGGTGGCTCAATAAATTGGTGTGTTCAAGAAAGAGTGTATCTT
255  G G S D R G G F N K F G V F K K E V Y L
901  CATACTACCAACACCTGAAAGCAGATGTGTTTCCAGACTGATCCAATCCAGAGATG
275  H T S P H L K A D V L F Q T D P T A E M
961  GCAGCTGAGTCATTGCCTTTCTCCTCGGACACTGTCCAGCTGGGAGCTGGAAGCCTGG
295  A A E S L P F S F G T L S S W E L E A W
1021  TATGAGACCTGCAAGAGTCTGCTCTCAGATGAAATGGGGTACCTATGTTTCACCT
315  Y E D L Q E V L S S D E N G G T Y V S P
1081  CCTGGAATGAAGAGGAAGAAATCAAAATCTTCAACACTCTTGACCTGCTTCTCTGGCT
335  P G N E E E E S K I F T T L D P A S L A
1141  TGGCTGACTGAGGAGGAGCCAGAACAGCAGAGTCAACAGCAGCTCCAGAGCCCTCAC
355  W L T E E E P R G P A E V T S T S Q S P H
1201  TCTCAGATTCCAGTCAAGCTCCCTGGCTCAGGAGGAAGAGGAGGAAGCAAGGGAGA
375  S P D S S Q S S L A Q E E E E E D Q G R
1261  ACCAGGAACCGGAACAGAGTGGTCAATCCAGCCCGGGCTGGAAGCAGCAGCTGAAG
395  T R K R K Q S S A G R K Q G R M K
1321  GAGAAAGAACAGGAGAAATGAAGAAAGTGGCAGCAGCTGAAGAGAATGAACGGCTC
415  E K E Q E N E R K V A Q L A E E N E R L
1381  AAGCAGGAAATCAGCGCGCTGACAGGGAATAGAGGCGACTCGCGGAGCTGCTATTGAC
435  K Q E I E R L T R E V E A T R R A L I D
1441  CGAATGGTGAATCTGACCAAGCATGAACATGGGAGCATCAGTCCCCACTTGGGCCA
455  R M V N L H Q A * 462
1501  CACTACCCACCTTTCCAGAGTGGCTACTGACTACCTCTCACTAGTCCCAATGATGTG
1561  ACCCTCAATCCACATACGACAGGGGGAAGGCTGGAGTAGACAAAGGAAGGCTCAGC
1621  TTGTATATAGAGATTGTACATTTATTATTACTGCTCCCTATCTATTAAAGTACTTCTTA
1681  TG (A)n 1682

```

TLS, COOH-terminus

b

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266  (<--TLS | CHOP -->)
      GCCCTCGGGACCAAGGATCACGT
      G P R D Q G S R
901  CATGACTCCGAACAGGATAATTGACAGCAACCAACCATCTTTGTGCAAGGCTGGGTGAG
275  H D S E Q D N S D N N T I F V Q G L K
961  AATGTTACAATTGAGTCTGTGGCTGATTACTTCAAGCAGATTGGTATTATTAAGACAAAC
295  N V T I E S V A D Y F K Q I G I I K T N
1021  AAGAAAACGGGACAGCCATGATTATTTGTACACAGCAGGAAATGGCAAGCTGAAG
315  K K T G Q P M I N L Y T D R F V Q G L K
1081  GGAGAGGCAACGGTCTCTTTTGTAGACCACCTTCAGTAAAGCAGCTATTGACTGGTTT
335  G E A T V S F D D P P S A K A A I D W F
1141  GATGGTAAAGAAATCTCCGGAATCTATCAAGGTCTCATTGTCTACTCGCGGGCAGAC
355  D G K E F S G N P I N L Y T D R F V Q G L K
1201  TTTAATCGGGTGGTGGCAATGGTCTGGAGGCGGAGGGGAGGAGGACCATGGGCGGT
375  F N R G G G N G R G G R G G G P M G B
1261  GGAGGCTATGAGAGTGGTGGCAGTGGTGGTGGTGGCGAGGAGGATTCCTCAGTGGAGT
395  G G Y G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G G
1321  GGTGGCGGTGGAGGACAGCAGCAGCTGGTACTGGAAGTGTCTAATCCCACTGTGAG
415  G G G G G G Q Q R A G D W K C P N P T C E
1381  AATATGAATCTCTTGGAGGAATGAATGAACAGCTGAAGGCCCTAAACAGATGGC
435  N M N F S W R N E C N Q K R G F P R R A D
1441  CCAGGAGGGGAGCAGGTGGTCTCAGTGGGGGTAACACGGGGATGATCGTCTGGT
455  P G G G P G G S H M G G N Y G D D R R G
1501  GGCAGGAGGAGCTATGATCGAGGCGGTACCGGGGCGCGGGGAGCGGTGGAGGCTTC
475  G R G Y D R G G G G G G G G G G G G G G G G G G G G G G G G G G G G
1561  CGAGGGGCGGGGTGGTGGGACAGAGTGGTCTTGGCCCTGGCAAGATGGATTCCAGG
495  R G G R G G G D R G G F G P G K M D S R
1621  GGTGAGCAGACAGGATCGCAGGAGAGGCGGTATTAATAGCTGGCTCCCAAGGTTTC
515  G E H R Q D R R E R P Y * 526
1681  TGGAAACAGCTTTTGTCTGTACCCAGTGTACCTCGTTATTTTGTAACTTCCAATTCT
1741  CTGATCACCAAGGGTTTCTTTTGTGTGAGTATGTAATTTGTAATATACCTCTGGTTC
1801  CCATTAAAGTGACCATTTTAGTT (A)n 1824

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FIG. 2 Nucleotide and predicted amino-acid sequence of TLS-CHOP and TLS. **a**, Sequence of the full-length TLS-CHOP cDNA isolated from the myxoid liposarcoma library. The junction between TLS and CHOP is indicated. The initiator methionine, normally used in the translation of CHOP, is in bold. **b**, The normal TLS sequence from the junction point with CHOP. The Arg-Gly-Gly tripeptide repeats in the C terminus of TLS are underlined. The numbering is continuous with that of TLS-CHOP.

METHODS. An unamplified λ -Zap (Stratagene) cDNA library constructed using mRNA from the myxoid liposarcoma cell line 1955/91 was hybridized to the murine CHOP cDNA. Over 40 independent clones encoding TLS-CHOP were isolated. The TLS portion of the TLS-CHOP cDNA was used as a probe to isolate clones encoding the normal TLS protein. Rapid amplification of cDNA ends (RACE¹⁵), with primers specific for TLS and TLS-CHOP was used to isolate the 5' ends of both transcripts and demonstrated that they are identical. The TLS-CHOP clone was shown to contain the full-length coding region by demonstrating that the cDNA could direct the expression, in COS-1 cells, of a protein indistinguishable in size from tumour TLS-CHOP (Fig. 3a). A variant form of TLS-CHOP, differing from the sequence shown here in 15 N-terminal residues was also isolated from the 1955/91 library. But this cDNA, when expressed in COS-1 cells, gave rise to a protein different in size from that found in the tumour cells, suggesting that it is a minor variant.

CHOP-reactive proteins from extracts of MLPS cells with a CHOP-specific antiserum and analysed them with the labelled dimerization domain of C/EBP β in a zipper blot assay⁵. The zipper probe readily recognizes the MMS-induced normal CHOP protein (29K) in both MLPS and control cells (Fig. 1b, lanes 2, 4 and 6). The MLPS cells contain, in addition to CHOP, a constitutive, larger protein (75K), that is immunoprecipitated by the CHOP antiserum and also reacts with the zipper probe (lanes 5–9). The 75K protein is nuclear (lane 10) and associates with other proteins (40–45K) that are also recognized by the C/EBP β zipper probe. This co-precipitating activity consists predominantly of C/EBP β which is the major dimerisation partner of CHOP (ref. 5, and data not shown). In conjunction

with the gene mapping information, localizing the breakpoints in *CHOP* to the 5' region of the gene³ these data indicate that MLPS cells contain a fusion protein, the C terminus of which is encoded by *CHOP* and includes its leucine zipper dimerization domain. The tumour-specific N-terminal extension is derived from another gene, provisionally named *TLS*.

We isolated clones encoding TLS-CHOP from an MLPS cDNA library. The 3' ends of all clones contained human CHOP sequence; however, upstream of the normal junction between CHOP exons 1 and 2, the sequence abruptly diverged from CHOP, confirming the presence of a tumour-specific fusion transcript (Fig. 2a). Reverse transcription of MLPS tumour RNA and PCR amplification of the junction between TLS and

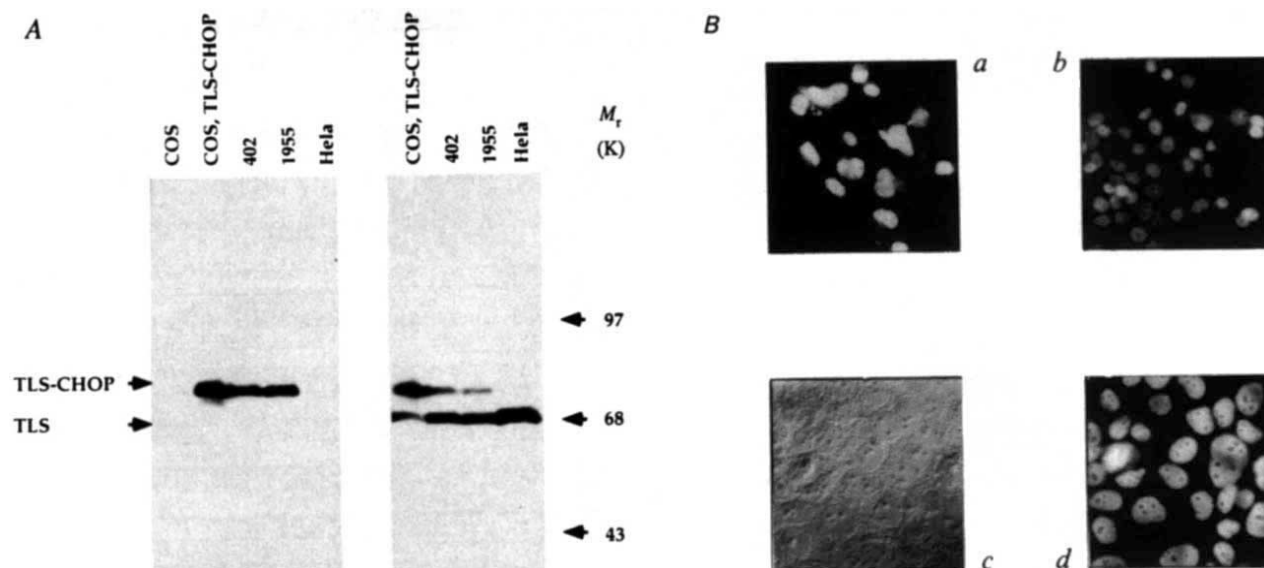


FIG. 3 TLS-CHOP and TLS are nuclear proteins. **A**, Western immunoblot of nuclear extracts from nontransfected COS-1 cells, COS-1 cells expressing the cloned TLS-CHOP, the myxoid liposarcoma cell lines 402/91 and 1955/91 and HeLa cells, probed with CHOP (left panel) and TLS (right panel) antisera. Both antisera recognize a 75K protein in the nuclear extracts of TLS-CHOP-containing cells. The TLS antiserum also recognizes a 68K protein, present in all cell lines. The position of the two proteins is indicated. The relatively faster migration on SDS-PAGE of the larger TLS protein, compared with the smaller TLS-CHOP is compatible with the previously recognized anomalous slow migration of CHOP⁵. **B**, Immunocytochemical localization of CHOP and TLS-CHOP. CHOP and TLS antisera stain the nucleus of 402/91 MLPS cells (*a* and *b*, respectively). In JEG-3 human choriocarcinoma cells that contain TLS but not TLS-CHOP, the TLS

antiserum stains the nucleus and spares the nucleoli, which appear as dark holes (*c*, Nomarski image and *d*, anti-TLS immune fluorescence). JEG-3 cells were chosen because of the good definition of nuclear morphology.

METHODS. A full-length expression plasmid for TLS-CHOP constructed in pCDNA-1 was transfected into COS-1 cells. Nuclear extracts and western immunoblots were as previously described⁵. Antisera were raised in rabbit against a full-length bacterially expressed CHOP-GST (glutathione *S*-transferase) fusion protein and a similar fusion protein containing the N-terminal portion of TLS (amino acids 78–244). The anti-CHOP antiserum and anti-TLS antiserum were used at a dilution of 1:1,000 as previously described⁵. Cells are shown at a magnification X400.

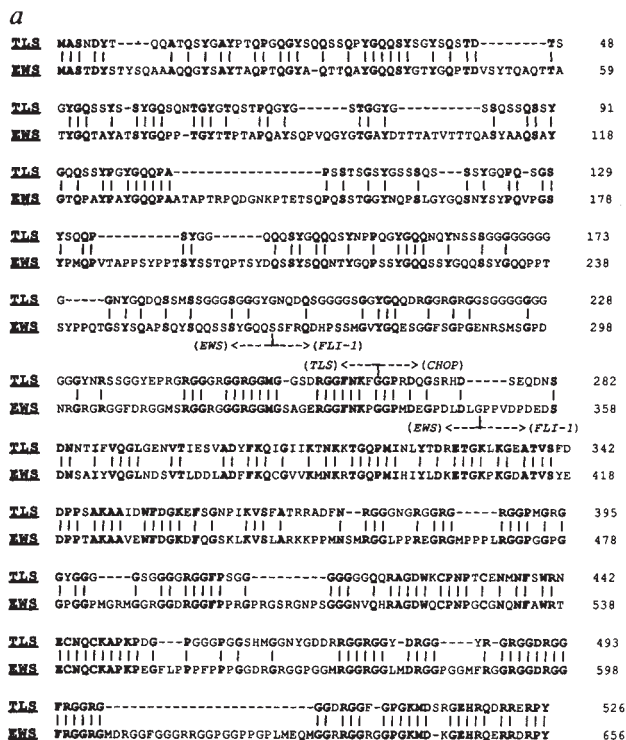
CHOP demonstrated the presence of a TLS-CHOP fusion transcript in all three cell lines and seven MLPS tumours tested and in none of the other liposarcomas tested (data not shown). The full-length fusion TLS-CHOP cDNA is similar in size to the abnormal CHOP transcript observed in the MLPS cells (Fig. 1*a*). It contains an uninterrupted open reading frame of 462 amino acids. The protein encoded by the fusion transcript consists of an N-terminal portion contributed by *TLS* and the full-length *CHOP* open reading frame in the C terminus. Linking them is a segment, translated only in the fusion mRNA, of the normal *CHOP* 5' untranslated region, between the beginning of *CHOP* exon 2 and its normal initiator methionine.

The cDNA library was rescreened for the full-length TLS cDNA. Several clones were isolated, the largest of which (Fig. 2*b*) is similar in size to the 1.9–2.0 kilobase (kb) mRNA present in variable amounts in all cell lines and tissues examined (data not shown) and contains an uninterrupted open reading frame of 526 amino acids. Antiserum against bacterially expressed TLS was used to probe western immunoblots containing nuclear extracts from MLPS cells. Both the CHOP antiserum and the TLS antiserum react with an indistinguishable 75K protein that is present in two different MLPS cell lines (Fig. 3*A*). In addition to the 75K TLS-CHOP the TLS antiserum also recognizes the normal *TLS* gene product, a 68K protein present in all cell lines tested. Immune fluorescent microscopy demonstrated that both TLS-CHOP, in MLPS cells and TLS, in normal cells, are nuclear proteins (Fig. 3*B*), as has been previously shown for CHOP⁵.

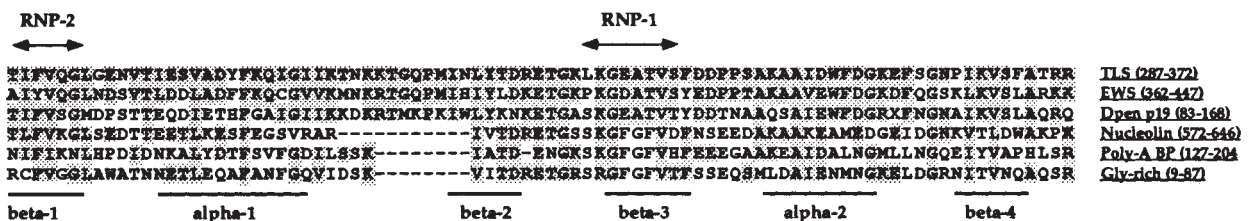
TLS has extensive sequence similarity (55.6% identity; Fig. 4*a*) to the recently described *EWS* gene, involved in a reciprocal balanced translocation, t(11;22)(q24;q12), that occurs in Ewing's sarcoma and other primitive neuro-ectodermal tumours⁷. In Ewing's sarcoma the N terminus of *EWS* is fused to the

DNA-binding domain of FLI-1, a member of the ETS family of transcription factors. The N terminus of *TLS* and *EWS* is extremely rich in the amino acids glutamine, threonine, serine, proline, tyrosine and glycine and contains multiple copies of the repeated hexapeptide: Ser/Gly-Tyr-Ser/Gly-Gln-Gln/Ser-Ser/Gln/Pro. In the C terminus, absent in the tumour-specific fusion proteins, both *TLS* and *EWS* contain a region of similarity to a conserved 80 amino-acid domain, the RNP-CS (ref. 8), found in several RNA-binding proteins, from species as distant as hamster and yeast (Fig. 4*b*). *TLS* and *EWS* both contain similar substitutions (Phe→Asp/Glu and Gly→Ala) in the highly conserved RNP-1 box and have an unusually large predicted loop structure between the α -helix-1 and β -sheet-2 of the RNP-CS. *TLS* and *EWS* share sequence similarity (52% identity) with the *Drosophila* protein Dpen p19 (ref. 9) and all three proteins appear to be members of a new sub-class of RNP-CS-containing proteins. In addition to the highly conserved RNP-CS motif in the C-terminus, both *TLS* and *EWS* contains multiple repeats of the Arg-Gly-Gly tripeptide, a sequence motif that has been implicated in RNA binding¹⁰.

Bacterially expressed full-length *TLS* and its C terminus both bind labelled RNA in a 'northwestern' ligand blot assay (Fig. 4*c*). Binding was enriched in the poly(A)⁺ fraction of the RNA, suggesting that the targets for *TLS* are in the messenger RNA fraction. The localization of *TLS* to nuclear regions outside the nucleolus (Fig. 3*B*, *d*) is also consistent with *TLS* binding a non-ribosomal RNA, perhaps to products of RNA polymerase II. Binding to poly(A)⁺ RNA may, however, simply reflect an increased affinity for homo-polymeric tracts of single-stranded nucleic acids. RNA binding by *TLS* maps to its region of similarity with other RNA-binding proteins. But the C-terminal fragment of *TLS* that retains RNA-binding activity is missing



b



c

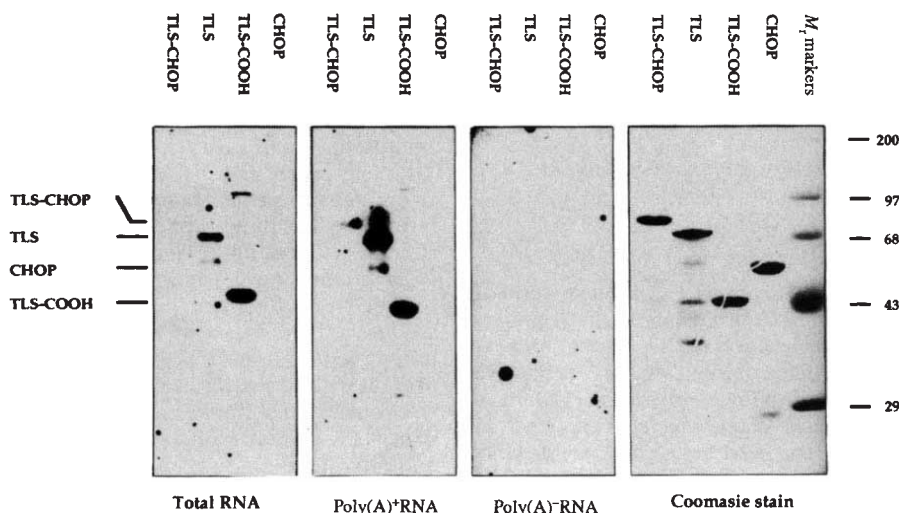


FIG. 4 **a**, Alignment of the amino-acid sequence of TLS and EWS. Vertical bars indicate identical residues. The position of the tumour-specific fusions with CHOP and FLI-1 are indicated. **b**, Alignment of the amino-acid sequences of TLS and other RNA-binding proteins in the region corresponding to the conserved RNP-CS domain⁸ in the human EWS gene product⁷, *Drosophila melanogaster* Dpen p19⁹, hamster nucleolin¹⁶, poly(A)⁺ binding protein from *Saccharomyces cerevisiae*¹⁷ and glycine-rich protein from sorghum¹⁸. The subdomains of the RNP-CS are indicated⁸. **c**, Left panel: northwestern blot of bacterially expressed proteins probed with either native, total cellular RNA, end labelled with ³²P or the similarly labelled poly(A)⁺ or poly(A)⁻ fractions. Right panel: photograph of the Coomassie-stained proteins. Arrows on the left and right point to the location of the proteins and molecular mass markers, respectively.

METHODS. TLS-CHOP and TLS were expressed in *Escherichia coli* fusion proteins with poly-histidine tag in the N terminus and purified by nickel-chelate chromatography. 'TLS-COOH' is a GST fusion protein containing amino acids 294–526 of TLS and 'CHOP' is a GST fusion protein containing the full-length murine CHOP sequence⁵. The purified proteins were resolved by 8% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked for 1 h in 20mM Tris-HCl, pH 7.9, 50 mM NaCl, 0.2mM EDTA, 1mM DTT, 1mM MgCl₂ containing 1% BSA and 20 µg ml⁻¹ yeast transfer RNA, and reacted, in the same buffer, with ~0.4 µg ml⁻¹ RNA that had been dephosphorylated with calf intestinal alkaline phosphatase and end-labelled with [³²P]γ-ATP, using T4 kinase (left panel). To demonstrate expression of all proteins an identical gel was stained with Coomassie blue (right panel).

several amino acids of the conserved RNP-2 portion of RNP-CS motif. It therefore appears likely that RNA-binding, measured in the northwestern blot assay, reflects also the activity of the dense cluster of Arg-Gly-Gly repeats in the C terminus of the molecule, perhaps working in conjunction with the RNP-CS motif. The presence of several similar tripeptide repeats in the N-terminal portion of TLS may account for the weak RNA-

binding activity of TLS-CHOP (visible on longer exposure of the blot shown in Fig. 4c).

In TLS-CHOP the RNA-binding portion of TLS is replaced by CHOP DNA-binding sequence. CHOP is transcriptionally silent in continuously dividing cells⁴, it is induced, however, in most cells in response to DNA-damaging agents⁴ and in fibroblasts during their differentiation *in vitro* to adipocytes⁵.

The coincidental induction of CHOP in a variety of situations associated with growth arrest suggests a role for the protein in control of cellular proliferation. The portion of TLS that is present in the TLS-CHOP fusion protein contains a glutamine-rich region similar to that found in transcriptional activation domains of regulatory proteins such as SP-1 (ref. 11) and a glycine-rich region, similar to that found in other RNA-binding proteins¹². We speculate that the portion of TLS that is present in TLS-CHOP serves an important effector function in the normal RNA-bound TLS, perhaps playing a role in transcriptional regulation. In TLS-CHOP, addition of this portion of TLS to the full-length CHOP protein may serve to convert a transcription factor involved in growth arrest to one associated with abnormal cellular proliferation. Inappropriate targeting of this domain of TLS to regulatory elements of specific genes by the DNA-binding and dimerization domain of CHOP may be important in the formation of myxoid liposarcoma. The regulation of adipocyte-specific genes by C/EBP proteins¹³ and the association of CHOP with both adipogenesis⁵ and the response to nutritional alterations in pre-adipocytes¹⁴, suggests that TLS-CHOP may regulate genes involved in adipose tissue-specific tumorigenesis. Fusion of the homologous N terminus of EWS to the DNA-binding domain of FLI-1 (ref. 7) suggests a conservation of function in the transforming activity present in both tumours. Ewing's sarcoma and MLPS are the first human solid tumours containing well defined chromosomal translocations in which the involved genes have been identified. The discovery of structurally related proteins in both cases suggests that juxtaposition of an effector domain from this new sub-class

of RNA-binding proteins with the DNA-binding domain of other transcription factors may represent a novel molecular mechanism for tumour formation common to several solid tumours. □

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1. Turc-Carel, C. et al. *Cancer Genet. Cytogenet.* **23**, 291–299 (1986).
2. Sreekantiah, S., Karakousis, C. P., Leong, S. P. L. & Sandberg, A. A. *Cancer* **69**, 2484–2495 (1992).
3. Åman, P. et al. *Genes Chrom. Cancer* **5**, 271–277 (1992).
4. Fornace, A. J. et al. *Molec. cell. Biol.* **9**, 4196–4203 (1989).
5. Ron, D. & Habener, J. F. *Genes Dev.* **6**, 439–453 (1992).
6. Park, J. S. et al. *Gene* **116**, 259–267 (1992).
7. Delattre, O. et al. *Nature* **359**, 162–165 (1992).
8. Kenan, D. J., Query, C. C. & Keene, J. D. *Trends biochem. Sci.* **16**, 214–220 (1991).
9. Haynes, S. R., Rebbert, M. L., Mozer, B. A., Forquignon, F. & Dawid, I. B. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1819–1823 (1987).
10. Kiledjian, M. & Dryfuss, G. *EMBO J.* **11**, 2655–2664 (1992).
11. Mitchell, P. J. & Tjian, R. *Science* **245**, 371–378 (1989).
12. Ghisolfi, L., Joseph, G., Amalric, F. & Erard, M. *J. biol. Chem.* **267**, 2955–2959 (1992).
13. Cao, Z., Umek, R. M. & McKnight, S. L. *Genes Dev.* **5**, 1538–1552 (1991).
14. Carlson, S. G., Fawcett, T. W., Bartlett, J. D., Bernier, M. & Holbrook, N. J. *Molec. cell. Biol.* (in the press).
15. Frohman, M. A., Dush, M. K. & Martin, G. R. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8998–9002 (1988).
16. Bugler, B. et al. *J. biol. Chem.* **262**, 10922–10925 (1987).
17. Sachs, A. B., Bond, M. W. & Kornberg, R. D. *Cell* **45**, 827–835 (1986).
18. Cretin, C. & Puigdomenech, P. *Pl. molec. Biol.* **15**, 783–785 (1990).

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A TCP1-related molecular chaperone from plants refolds phytochrome to its photoreversible form

Eckart Mummert*, Rudolf Grimm*†, Volker Speth*, Christoph Eckerskorn‡, Emile Schiltz§, Anthony A. Gatenby|| & Eberhard Schäfer*

*Institut für Biologie 2, Schänzlestrasse 1, D-7800 Freiburg, Germany

‡Max-Planck-Institut für Biochemie, Genzentrum, D-8033 Martinsried, Germany

§Institut für organische Chemie und Biochemie, Albertstrasse 21, D-7800 Freiburg, Germany

||Molecular Biology Division, Central Research and Development, E.I. DuPont de Nemours and Company, Wilmington, Delaware 199880–0402, USA

FOLDING of the major cytoskeletal components in the cytosol of mammalian cells is mediated by interactions with t-complex polypeptide-1 (TCP1) molecular chaperones^{1–6}, a situation analogous to the chaperonin 60-aided folding of polypeptides in bacteria^{7,8}, chloroplasts^{9,10} and mitochondria^{11–13}. We have purified a TCP1-related molecular chaperone from etiolated oat seedlings that has a unique structure. Although immunologically related to TCP1, and having amino-acid sequence similarity, its quaternary structure is different from animal TCP1 proteins^{5,6,14}. Electron microscopy and image analysis reveals that the chaperone has two stacked rings of six subunits each, and is distinct in size and configuration. The chaperone copurifies with the soluble cytosolic photoreceptor phytochrome¹⁵, and can stimulate refolding of denatured phytochrome to a photoactive

form in the presence of Mg-ATP. We propose that this protein is the cytosolic chaperone involved in phytochrome biogenesis in plant cells.

Phytochrome is a cytosolic plant photoreceptor that exists in two forms that are interconvertible by light¹⁵. The physiologically inactive P_r form ($\lambda_{\max}$ 666 nm) can be transformed to the functional P_{fr} form ($\lambda_{\max}$ 730 nm) by red light, resulting in the specific activation and repression of many light-dependent genes, and concomitant morphogenesis^{15,16}. During the isolation of phytochrome from etiolated oat (*Avena sativa*) seedlings, an oligomeric protein containing 60K polypeptides copurified with the photoreceptor (Fig. 1a). This polypeptide has limited crossreaction with antisera raised against pea (*Pisum sativum*) chloroplast chaperonin 60 (cpn60) (Fig. 1b), and microsequencing gave the amino-terminal sequence Ala-Leu-Glu-Ser, which has some homology to the chloroplast cpn60 α -subunit of *Ricinus communis*⁹.

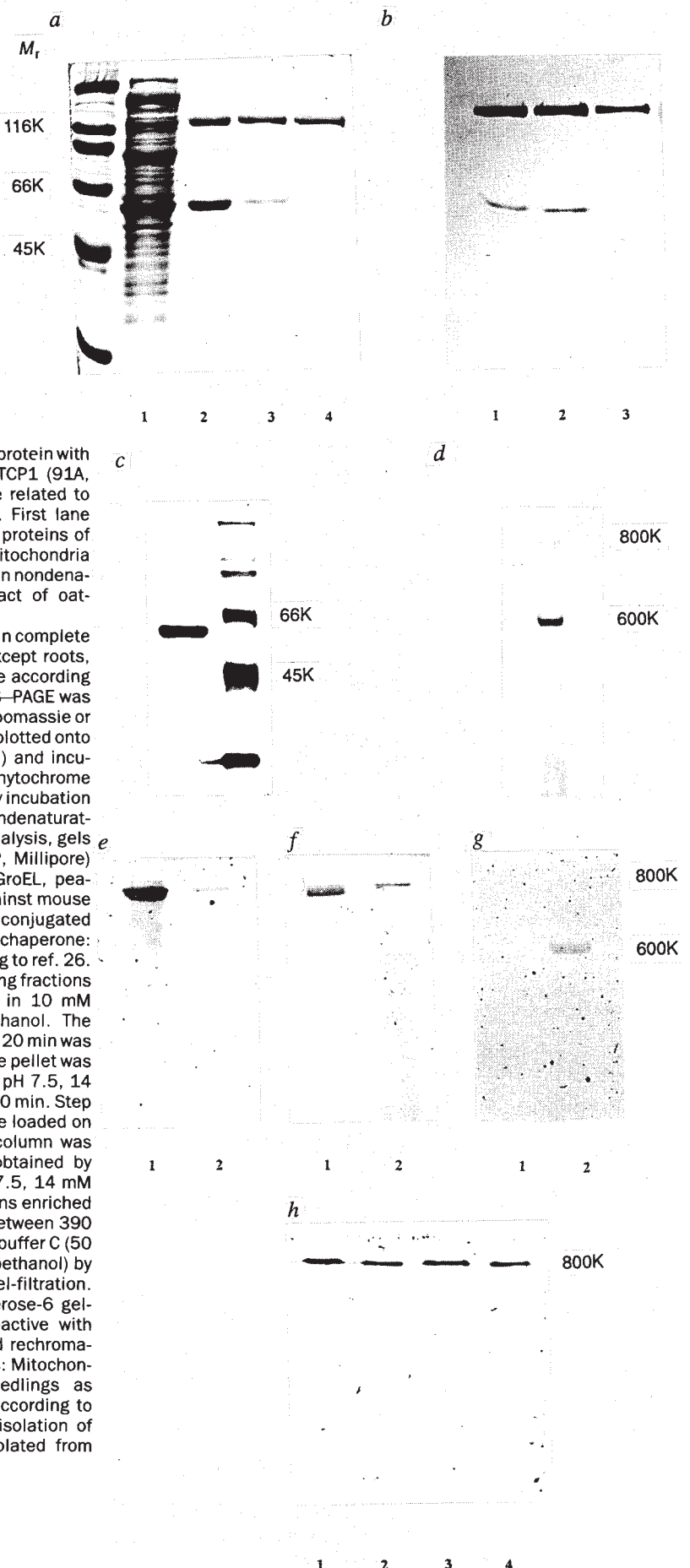
We purified the protein to homogeneity as judged by silver staining of sodium dodecyl sulphate (SDS) gels (Fig. 1c). Nondenaturing gel electrophoresis of the purified protein revealed a predominant oligomer of about 600K, and traces of a second protein of about 800K (Fig. 1d). Immunoblotting of non-denaturing gels revealed an interaction of the minor 800K protein with antisera raised against *Escherichia coli* GroEL or chloroplast cpn60, but only a weak interaction of the predominant 600K protein with anti-chloroplast cpn60, and none with anti-GroEL (Fig. 1e, f). In contrast, if the immunoblots were prepared using monoclonal antibodies raised against mouse TCP1, the major 600K protein crossreacted strongly, but the minor 800K species and the bacterial GroEL marker did not interact (Fig. 1g). There were similar patterns of cross reactivity with various antisera when the protein bands were excised from non-denaturing gels, resolved on SDS gels and immunoblotted (data not shown). The 600K protein is, therefore, immunologically related to TCP1. The 800K protein probably originates from contaminating mitochondria or plastids. To confirm this, chloroplast, etioplast and mitochondrial fractions were prepared

† To whom correspondence should be addressed.

‡ Present address: Hewlett Packard GmbH, Analytische Messtechnik, D-7517 Waldbronn 2, Germany.

FIG. 1 Purification and characterization of the oat chaperone. *a*, Coomassie stained SDS-PAGE revealing copurification of a 60K fraction with phytochrome. Lanes: 1, supernatant after washing the $(\text{NH}_4)_2\text{SO}_4$ precipitated pellet of the phytochrome-containing fraction after hydroxylapatite column chromatography with buffer A (10 mM KP_i pH 7.8, 14 mM 2-mercaptoethanol); 2, supernatant after washing the pellet of the previous step with buffer B (100 mM KP_i pH 7.8, 14 mM 2-mercaptoethanol); 3, supernatant after washing the previous pellet with buffer A; 4, solubilized pellet of the PVP 40000 precipitation of the supernatant shown in lane 3. *b*, Western blot analysis using anti-oat-phytochrome antisera (pAVR), followed by anti-pea-chloroplast cpn60 antisera shows crossreaction of the 60K polypeptide with the latter. Lane 1, 2 and 3 correspond to lanes 2, 3 and 4, respectively, in *a*. *c*, Purified 60K fraction in silver-stained SDS gels. *d*, Nondenaturing gels indicate that its oligomeric structure is different from TCP1 or cpn60. *e*, *f*, *g*, Immunoblot analysis of the 60K fraction after nondenaturing PAGE using anti-GroEL (*e*), anti-chloroplast cpn60 (*f*) or anti-TCP1 (*g*) antisera showing weak crossreaction of the 600K protein with anti-chloroplast cpn60 and strong interaction with anti-TCP1 (91A, provided by K. Willison). The traces of 800K protein are related to cpn60 and are not recognized by anti-TCP1 antibodies. First lane GroEL, second lane oat-chaperone on every blot. *h*, Only proteins of about 800K from purified etioplasts, chloroplasts and mitochondria exhibit crossreaction with anti-chloroplast cpn60 antisera in nondenaturing immunoblots. Lanes: 1, GroEL; 2, crude extract of oat-etioplasts; 3, chloroplasts; and 4, mitochondria.

METHODS. Oat seedlings were grown on moist vermiculite in complete darkness for 4½ days at 25 °C. Whole oat seedlings, except roots, were harvested. Purification of oat phytochrome was done according to ref. 26. Electrophoresis and western blot analysis: SDS-PAGE was done according to Laemmli²⁷ using 10% gels. Gels were Coomassie or silver-stained. For western blot analysis, gels were electroblotted onto nitrocellulose membranes (0.22 µm, Schleicher&Schuell) and incubated with polyclonal antisera (pAVR) raised against oat-phytochrome or chloroplast cpn60 from pea (*Pisum sativum*), followed by incubation with alkaline phosphatase-conjugated anti-rabbit IgG. Nondenaturing PAGE was done²⁷ using 7.5% gels. For immuno-blot analysis, gels were electroblotted onto PVDF membranes (Immobilon P, Millipore) and incubated with polyclonal antisera raised against GroEL, pea-chloroplast cpn60 or monoclonal antisera (91A) raised against mouse TCP1³ followed by incubation with alkaline phosphatase conjugated anti-rabbit or anti-rat IgG, respectively. Purification of oat chaperone: Step 1. Purification of oat phytochrome was done according to ref. 26. After $(\text{NH}_4)_2\text{SO}_4$ -precipitation of the phytochrome-containing fractions of the hydroxylapatite column the pellet is solubilized in 10 mM KP_i-buffer (pH 7.8) containing 14 mM 2-mercaptoethanol. The supernatant of the following centrifugation at 49,000 g for 20 min was subjected to a 42% saturated $(\text{NH}_4)_2\text{SO}_4$ -precipitation. The pellet was redissolved in equal volume of buffer A (20 mM Tris-HCl pH 7.5, 14 mM 2-mercaptoethanol) and centrifuged at 49,000 g for 20 min. Step 2. Ion-exchange chromatography. Supernatant (2 ml) were loaded on an anion-exchange column (Mono Q, Pharmacia). The column was washed with 5 ml buffer A. Elution of proteins was obtained by increasing the amount of buffer B (20 mM Tris-HCl pH 7.5, 14 mM 2-mercaptoethanol, 1 M NaCl) from 0 to 50%. The fractions enriched in reactivity to anti-chloroplast cpn60 antiserum eluted between 390 and 450mM NaCl and were concentrated and dissolved in buffer C (50 mM KP_i-buffer pH 7.5, 150 mM NaCl, 14 mM 2-mercaptoethanol) by ultrafiltration with centricon-10 tubes (Amicon). Step 3. Gel-filtration. Concentrated fractions (200 µl) were loaded on a Superose-6 gel-filtration column (Pharmacia). The fractions immunoreactive with anti-chloroplast cpn60 antiserum were concentrated and rechromatographed on the same column. Preparation of organelles: Mitochondria were isolated from 5-day-old etiolated oat seedlings as described²⁸. Chloroplasts and etioplasts were purified according to ref. 29. For etioplasts the same seedlings as for the isolation of mitochondria were used whereby chloroplasts were isolated from 6-day-old seedlings grown in continuous white light.



a A L V P W W I P P
A L E S K G G
G Q K K P Q Q V K

b A L Q S G I D K L

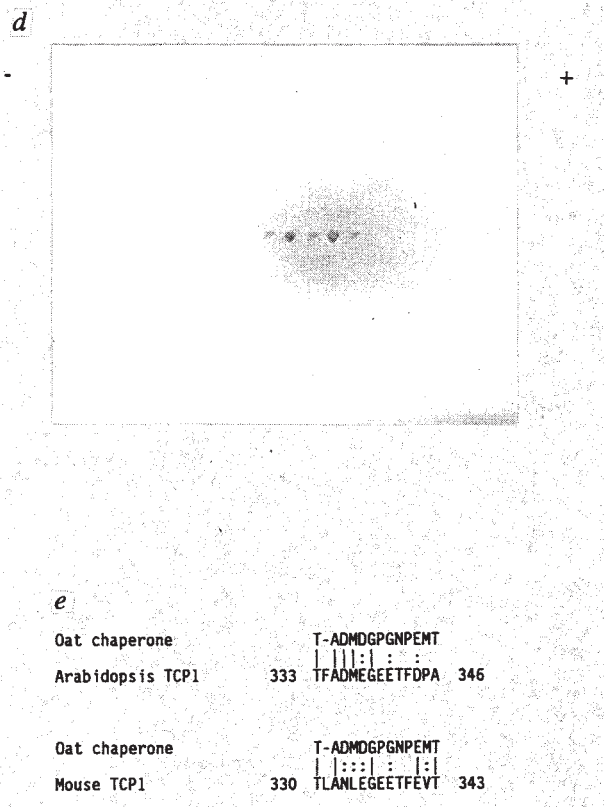


FIG. 2 Sequencing, two-dimensional electrophoresis and limited proteolysis of the oat chaperone. *a*, N-terminal sequence (single-letter amino-acid code) of the 60K fraction revealing strong heterogeneity after the fourth amino acid compared to *b*, the N-terminal sequence of the α -subunit of chloroplast cpn60 from *Ricinus communis*. *c*, The 60K fraction splits up into six polypeptides with an isoelectric point between 5 and 6 in two-dimensional electrophoresis. *d*, Five of the six protein spots show crossreaction with anti-TCP1 antibodies (91A) in western blots. *e*, The sequence of a 15K peptide generated by limited proteolysis with proteinase Glu-C exhibits similarity to internal sequences of TCP1 from *Arabidopsis*¹⁷ and mouse¹⁸.

METHODS. Two-dimensional electrophoresis. Oat chaperone was analysed by two-dimensional PAGE using an immobilized pH-gradient between pH 4 and 7 (Immobiline dry strip, Pharmacia) in the first dimension and a vertical SDS-PAGE (ExcelGel, Pharmacia) in the second dimension according to the manufacturer's manual. The proteins were then transferred onto a PVDF membrane (Immobilon P, Millipore) which was Coomassie stained or incubated with anti-TCP1-

from oat seedlings, electrophoresed on nondenaturing gels, and incubated with anti-chloroplast cpn60. Only the 800K protein was observed (Fig. 1*h*), with no trace of the 600K protein, even though the latter can weakly interact with anti-chloroplast cpn60 (Fig. 1*f*). The 600K protein was also absent in purified nuclei (data not shown), and therefore it is probably located in the cytosol, although a definitive answer will require immunolocalization studies.

Microsequencing of the 60K polypeptides gave an N-terminal sequence with some homology to *R. communis* chloroplast cpn60⁹, but is highly divergent after the fourth amino acid (Fig. 2*a, b*). This nonhomogeneity of the sequence suggests that at least three different polypeptides are present that have identical amino acids (Ala-Leu-Glu-Ser) at their N terminus. This is reinforced by six distinct species with isoelectric points between 5 and 6 found after two-dimensional electrophoresis of the 60K polypeptides (Fig. 2*c*), and five of the six species sequenced all possessed Ala-Leu-Glu-Ser at the N terminus. This heterooligomeric structure is found for TCP1 particles from mammalian cells^{5,6,14}, and also appears to be a feature of the oat chaperone. Immunoblots of the two-dimensional gels revealed



antibodies(91A) followed by incubation with alkaline phosphatase-conjugated anti-rat-IgG. Microsequencing. Oat chaperone was subjected to SDS-PAGE or two-dimensional electrophoresis with a pH-gradient between pH 4 and 9, respectively, according to ref. 30. The gels were blotted on a polypropylene membrane (Selex 20, Schleicher&Schuell). After Coomassie staining and drying, the 60K band and the protein spots, respectively, were excised. N-terminal amino-acid sequence was analysed on an Applied Biosystems 470A gasphase-sequencer as described³¹. Limited proteolysis and microsequencing of a 15K fragment. Oat chaperone (100 μ g) was incubated with 2.5 μ g proteinase Glu-C (Sigma) in 100 μ l KP_i-buffer (pH 7.8) for 24 h at room temperature. The solution was subjected to SDS-PAGE²⁷, blotted onto a PVDF membrane (Immobilon P, Millipore) and Coomassie stained. A protein band of about 15K was excised and N-terminal sequenced in an Applied Biosystems sequencer 477A/analyser 120A-system according to the manufacturer's manual.

that five of the six polypeptides crossreact with anti-TCP1 (Fig. 2*d*). Furthermore, sequencing of an internal protease-generated 15K fragment gave sequence similarities of 62 and 77% when compared to the predicted TCP1 proteins from *Arabidopsis*¹⁷ and mouse¹⁸, respectively (Fig. 2*e*). Electron micrographs of the 600K chaperone demonstrate a unique structure. Image analysis reveals that the oat chaperone has a double toroid structure, but with six subunits present in each of the two rings (Fig. 3*a, b*). This is very different from the 7-fold rotational symmetry of the cpn60 chaperones of chloroplasts, mitochondria and bacteria^{7,8,12,19}, and also contrasts with the double rings of 8 or 9 subunits for other TCP1-related proteins isolated from mammals and archaeobacteria^{5,6,20,22}. This difference in subunit composition is also supported by the M_r determined using size exclusion chromatography (data not shown) and on nondenaturing gels (Fig. 1*b*).

A characteristic feature of molecular chaperones is that they couple the hydrolysis of ATP to protein folding^{19,21}. We measured the ability of the purified oat chaperone to hydrolyse ATP, and to aid protein folding. ATPase was assayed²³ at 25 °C and gave activities of 0.25 mol ATP s⁻¹mol⁻¹ for the oat

chaperone, compared to $0.5 \text{ mol ATP s}^{-1}\text{mol}^{-1}$ for GroEL. Because the oat chaperone was isolated after its copurification with phytochrome, we examined its influence on refolding of the photoreceptor by measuring photoreversibility between the P_r and P_{fr} forms. Phytochrome is a homodimer with 124K subunits, each attached to a tetrapyrrolic chromophore²⁴. Chemically denatured phytochrome (P-U) does not refold spontaneously after dilution from guanidine-HCl under the conditions tested²⁵. When P-U was diluted into refolding buffer containing the oat chaperone, photoreversibility was also not observed, until Mg-ATP was added (Fig. 4a). Therefore, successful refolding of photoactive phytochrome can be aided by a molar excess of the 600K chaperone and Mg-ATP. Phytochrome reconstitution could also be examined using a monoclonal antiserum (MAC56) that fails to interact with denatured phytochrome, but does recognize the native protein (data not shown). The appearance of phytochrome that can interact with MAC56 occurs under the same conditions that phytochrome photoreversibility is observed (Fig. 4b).

Several lines of evidence indicate that oat cells contain a hetero-oligomeric molecular chaperone that exhibits some similarities to mammalian TCP1, the most noteworthy being its partial amino-acid sequence homology and its crossreaction with anti-TCP1. The oat protein shares in common with other chaperones the ability to refold denatured proteins, and the possession of an ATPase activity. In contrast to other chaperones of the TCP1 and cpn60 types, the oat protein has a unique size (600K) and structural arrangement of two rings of six subunits. The 600K chaperone is not localized in plastids or mitochondria, and its copurification with a cytosolic protein indicates that it is probably a cytosolic chaperone involved in the biogenesis of phytochrome. This does not exclude a role for the 600K chaperone in the folding of other plant proteins, especially as it appears to be an abundant protein (E.M., unpublished data). In addition to its observed ability to assist phytochrome

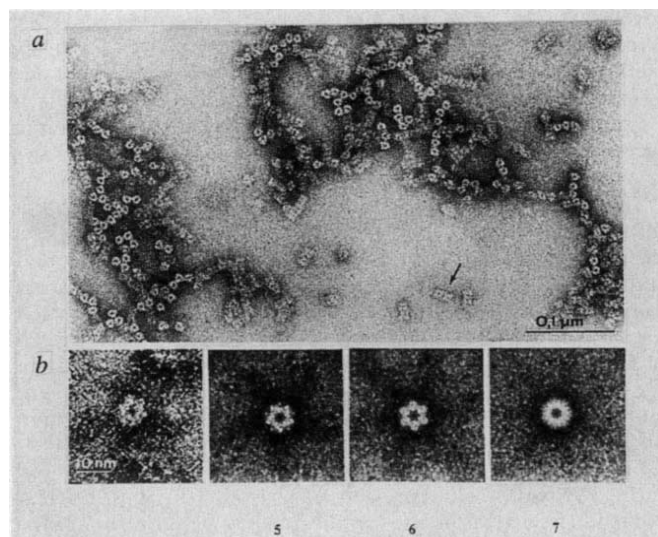


FIG. 3 Electron micrographs of the 600K chaperone. *a*, Chaperone oligomers in side and top view. The arrow points at a row of oligomers in side view. *b*, A single oligomer in top view, followed by its Markham analysis, revealing its six-membered ring structure. Numbers indicate the number of circular shifts per 360° .

METHODS. Negative stain of the protein solution was obtained with 1% aqueous uranyl acetate on carbon-coated 300-mesh copper grids, rendered hydrophilic by glow discharge. The samples were examined at 80 kV in a Philips CM 10 transmission electron microscope. The number of subunits of the oat chaperonin was determined using the rotation analysis method of Markham³².

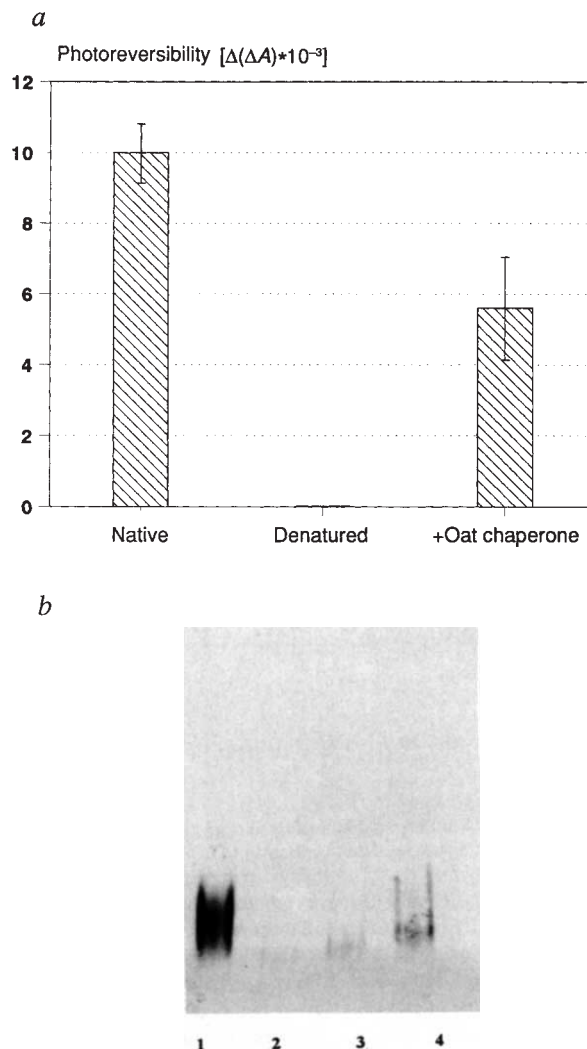


FIG. 4 Refolding of denatured oat phytochrome. *a*, Plot of the photoreversibility (in $\Delta(AA)$ values) measured in the 'Ratiospect' spectro-photometer showing restoration of photoreversibility with oat chaperone. *b*, Immunoblot after nondenaturing PAGE using monoclonal anti-phytochrome antiserum MAC56 (provided by B. Thomas). Lanes: 1, native phytochrome; 2, 3, 4, refolding products obtained with a molar excess of oat chaperone to phytochrome of 1.5:1 (2), 3:1 (3) or 6:1 (4), respectively.

METHODS. Refolding assay. Purified oat phytochrome was denatured for 1 h at 25°C in a buffer of 100 mM Tris-HCl pH 7.8, 6M guanidine hydrochloride, 1 mM 2-mercaptoethanol. The final concentration of denatured phytochrome (P-U) was $8 \mu\text{M}$ protomer. The refolding assay was done at 4°C in a final volume of 500 μl . Refolding of phytochrome was followed either in the absence or presence of oat chaperone, Tween-20 or BSA. To investigate spontaneous refolding, 5 μg of P-U were rapidly diluted with mixing into cold refolding buffer (100 mM Tris-HCl pH 7.8, 10 mM MgCl_2 , 10 mM KCl, 1mM-2-mercaptoethanol) without additional components or containing 0.01% Tween 20 or 6 μM BSA. For assisted refolding oat chaperone was diluted into cold refolding buffer. Final concentrations of oat chaperone oligomer were 60 nM, 120 nM and 240 nM. After 5 min, 5 μg of P-U corresponding to a final concentration of 80 nM protomer was added. Refolding reaction was started after another 5 min by adding ATP pH 7.8 to a final concentration of 2 mM. After 1 h restoration of photoreversibility of phytochrome was measured in a modified dual wavelength 'Ratiospect' spectrophotometer³³ using CaCO_3 as a light-scattering agent. Electrophoresis and western blotting. Nondenaturing PAGE was as in Fig. 1. For detection of native and refolded phytochrome, blots were incubated with monoclonal antibody MAC56 followed by alkaline phosphatase conjugated anti-rat IgG. Although a precise epitope mapping for MAC56 is not available yet, the binding region of the antibody is not located in the first 10K of oat phytochrome (B. Thomas, personal communication).

refolding, possible interactions of the 600K chaperone in stabilization of the two physiologically important P_r and P_{fr} conformations, and the rapid sequestering of P_{fr} after red-light irradiation¹⁵ can now be investigated. □

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1. Ellis, R.J. *Science* **250**, 954–959 (1990).
2. Gupta, R.S. *Biochem. Int.* **4**, 833 (1990).
3. Willison, K. *et al. Cell* **57**, 631–632 (1989).
4. Yaffe, M.B. *et al. Nature* **358**, 245–248 (1992).
5. Lewis, V.A., Hynes, G.M., Zheng, D., Saibil, H. & Willison, K. *Nature* **358**, 249–252 (1992).
6. Frydman, J. *et al. EMBO J.* **11**, 4767–4778 (1992).
7. Hendrix, R.W. *J. molec. Biol.* **129**, 375–392 (1979).
8. Gatenby, A.A. & Ellis, R.J. *Rev. Cell Biol.* **6**, 125–149 (1990).
9. Hemmingsen, S.M. *et al. Nature* **333**, 330–334 (1988).
10. Hemmingsen, S.M. & Ellis, R.J. *Pl. Physiol.* **80**, 269–276 (1986).
11. McMullin, T. & Hallberg, R.L. *Molec. cell. Biol.* **8**, 371–380 (1988).
12. Hallberg, R.L. *Semin. Cell Biol.* **1**, 37–45 (1990).
13. Jindal, S., Dudani, A.K., Singh, B., Harley, C.B. & Gupta, R.S. *Molec. cell. Biol.* **9**, 2279–2283 (1989).
14. North, G. *Nature* **354**, 434–435 (1991).
15. Furuya, M. *Adv. Biophys.* **25**, 133–167 (1989).
16. Gilmartin, P.M., Sarokin, L., Memelink, J. & Chua, N.-H. *Pl. Cell* **2**, 369–378 (1990).

17. Mori, M. *et al. Gene* **122**, 381–382 (1992).
18. Kubota, H. *et al. Gene* **120**, 207–215 (1992).
19. Ellis, R.J. & van der Vies, S.M. *A. Rev. Biochem.* **60**, 321–347 (1991).
20. Trent, J.D., Nimmersgern, E., Wall, J.S., Hartl, F.-U. & Horwich, A.L. *Nature* **354**, 490–493 (1991).
21. Viitanen, P.V. *et al. Biochemistry* **29**, 5665–5671 (1990).
22. Phipps, B.M. *et al. Nature* **361**, 475–477 (1993).
23. Lill, R., Dowhan, W. & Wickner, W. *Cell* **60**, 271–280 (1990).
24. Rüdiger, W., Thümmler, F., Cmiel, E. & Schneider, S. *Proc. natn. Acad. Sci. U.S.A.* **80**, 6244–6248 (1983).
25. Grimm, R., Donaldson, G.K., van der Vies, S.M., Schäfer, E. & Gatenby, A.A. *J. biol. Chem.* **268**, 5220–5226 (1993).
26. Grimm, R. & Rüdiger, W.Z. *Naturforschung* **41c**, 988–992 (1986).
27. Laemmli, U.K. *Nature* **227**, 680–685 (1970).
28. Jackson, J.F. in *Modern Methods of Plant Analysis, Vol.1, Cell Components* (eds Linskens, H.F. & Jackson, J.F.) 301 (Springer, Berlin, 1985).
29. Pilwat, G., Hampp, R. & Zimmermann, U. *Planta* **147**, 396–404 (1980).
30. Gög, A., Postel, W. & Günther, S. *Electrophoresis* **9**, 531–546 (1988).
31. Eckerskorn, C., Mewes, W., Goretzki, H. & Lottspeich, F. *Eur. J. Biochem.* **176**, 509–519 (1988).
32. Markham, R., Frey, S. & Hills, G.J. *Virology* **20**, 88–102 (1963).
33. Gross, J., Seyfried, M., Fushanskyy, L. & Schäfer, E. in *Techniques in Morphogenesis* (eds Smith, H. & Holmes, M.G.) 131–158 (Academic, London, 1984).

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New eukaryotic transcriptional repressors

Shamol Saha*, Joshua M. Brickman, Norbert Lehming & Mark Ptashne

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, Massachusetts 02138, USA

TRANSCRIPTIONAL activating sequences have been described¹ that are encoded by parts of the genome of *Escherichia coli*. These acidic peptides, fused to a DNA-binding fragment of the yeast transcriptional activator GAL4, activate transcription of a gene in a wide array of eukaryotes, provided that gene bears GAL4-binding sites nearby^{2–4}. Here we describe an *E. coli*-encoded sequence that, when attached to the same DNA-binding fragment (GAL4(1–147)), converts that fragment into a repressor. Thus, as assayed in yeast or *in vitro* in yeast extracts, this molecule represses transcription when bound upstream of a variety of different activators. Two additional repressing regions that work when tethered upstream, a multiple mutant derivative of the original isolate and a synthetic peptide are, like the original isolate, highly basic. At least one activator can be inhibited by the mutant but not by the parental repressing region. These and other findings suggest that these repressing regions interact with and inhibit the activity of activating regions bound nearby on DNA.

Our new repressors were isolated as diagrammed in Fig. 1a. Figure 1b shows that one of these molecules, GAL4(1–147) + SSB24, repressed transcription when bound upstream of the yeast activator GCN4 (ref. 5). Constructs bearing five repressor binding sites some 72 base pairs (bp) upstream of one GCN4 site were repressed about 14-fold (line 1); increasing the number of activator sites decreased repression (line 2), and decreasing the number of repressor binding sites reduced the repression still more (line 3). Moving the repressor binding sites from 72 to 221 bp upstream of an activator site decreased repression about twofold (compare lines 1 and 4). At the larger distance of 500 bp, there was no repression (data not shown), nor was repression observed with a construct lacking GAL4-binding sites (line 5). Primer extension analysis (Fig. 1c) shows that the reduction in lacZ activity observed when GAL4(1–147) + SSB24 was bound upstream of GCN4 was correlated with a reduction in GAL1-lacZ transcription. Western blots of extracts made

from strains expressing both GAL4(1–147) and GAL4(1–147) + SSB24 show that these proteins were expressed at comparable levels (data not shown). In contrast to the effect of some native yeast repressors^{6,7}, repression by GAL4(1–147) + SSB24 did not require SSN6 function (data not shown). Figure 1 also shows that a second repressor isolated as described in Fig. 1a (GAL4(1–147) + SSB16) repressed less efficiently than did GAL4(1–147) + SSB24, whether it was bound upstream or downstream of the two GCN4 sites.

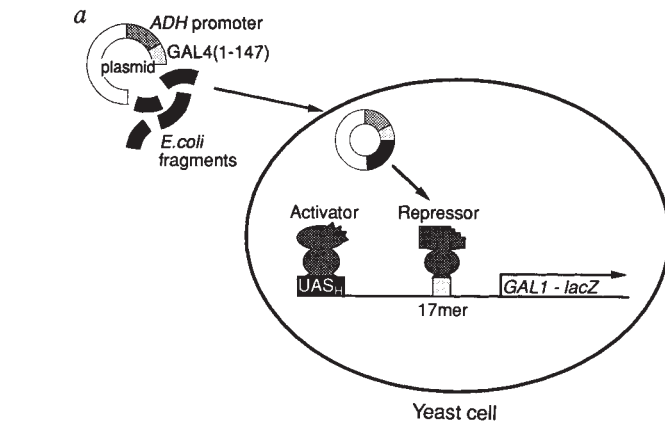
The three experiments shown in Fig. 1d demonstrate that, with a constant number of repressor binding sites, the weaker the activation, the greater the repression: (1) a weakly activating mutant allele of GCN4 (which binds DNA with an affinity comparable to that of the wild-type protein *in vitro*⁵) was repressed more efficiently than was wild-type GCN4 (80-fold versus 12-fold) (Fig. 1d, line 1); (2) repression was even stronger (over 200-fold) with the still weaker activator Dorsal^{8,9}, a *Drosophila* protein that activates the genes *twist* and *snail* in the fly embryo (Fig. 1d, line 2)^{10,11}; (3) weakening the activation by moving the position of the activator (in this case, wild-type GCN4) further upstream, but holding constant the distance between the activator and the repressors, increased repression (compare lines 3 and 1 in Fig. 1d).

The experiments shown in Fig. 2b and c demonstrate that a mutant of GAL4(1–147) + SSB24, termed GAL4(1–147) + SSBM7, repressed transcription stimulated by proteins bound to the *URA3* upstream activating sequence (UAS_U)¹² substantially more efficiently than did the parent: Fig. 2b shows the response of a reporter gene to repressor activity in cells grown in culture, and Fig. 2c shows the survival of cells on a medium that kills cells expressing the *URA3* gene. Figure 2a shows, in contrast, that GAL4(1–147) + SSB24 repressed transcription elicited by GCN4 more efficiently than did the mutant.

Figure 3 shows that GAL4(1–147) + SSB24, expressed in and purified from *E. coli*, repressed transcription in yeast nuclear extracts. The activators used in these experiments, also purified from *E. coli*, were LexA-B112 (ref. 13) and LexA-VP16. Figure 3a demonstrates that the weaker activator LexA-B112 was repressed more efficiently than was the stronger activator LexA-VP16; Fig. 3b shows that repression required the SSB24 repressing region and GAL4 sites in the template; and Figure 3c confirms the *in vivo* observation that, for a given repressor and activator, a higher ratio of repressor to activator binding sites results in greater repression.

The sequences of peptides tested for repressing activity are shown in Fig. 4. The most striking features of both peptides is their high degree of basicity: SSB24 and SSBM7 bear net charges

* Present address: Weis Center for Research, 100 North Academy Avenue, Geisinger Clinic, Danville, Pennsylvania 17822, USA.



		β-Galactosidase activity	
		one 17mer	two 17mers
	—	960	600
	GAL4(1-147)	640	190
	GAL4(1-147)+SSB24	30	10
	GAL4(1-147)+SSB16	104	23
	GAL4	8,410	16,800

b

		β-Galactosidase activity	
		one 17mer	two 17mers
1	—	840	
	GAL4(1-147)	450	
	GAL4(1-147)+SSB24	60	
	GAL4(1-147)+SSB16	400	
	GAL4(1-147)+Lys/Ala	95	
	GAL4(1-147)+poly Lys	200	
2	—	1890	
	GAL4(1-147)	1200	
	GAL4(1-147)+SSB24	400	
3	—	800	
	GAL4(1-147)	850	
	GAL4(1-147)+SSB24	400	
4	—	520	
	GAL4(1-147)	320	
	GAL4(1-147)+SSB24	70	
5	—	1220	
	GAL4(1-147)	1110	
	GAL4(1-147)+SSB24	1170	

d

		β-Galactosidase activity	
		WT GCN4	Mutant GCN4
1	—	1500	80
	GAL4(1-147)+SSB24	120	1
2	—	0.8	
	Dorsal	20	
	Dorsal + GAL4(1-147)+SSB24	<0.1	
3	—	44	
	GAL4(1-147)+SSB24	1.2	

FIG. 1 Repressor isolation and activities. **a**, Repressor isolation screen and activities of two candidates bound between the UAS of the *His4* gene (UAS_H) and a reporter gene. The top part of the panel shows the parental cell which expresses β-galactosidase at a high level and forms a blue colony on X-gal indicator plates. Cells containing GAL4(1-147) form lighter blue colonies, and the more efficient repressors, isolated at a frequency of 0.005% from the library in ref. 19, render the colonies white. The bottom part of the panel describes the activity of the two candidates GAL4(1-147) + SSB24 and GAL4(1-147) + SSB16 as assayed by the level of β-galactosidase in cultured cells bearing one of the indicated reporters. **b**, Upstream repression as a function of number and position of binding sites. Cells containing the indicated reporter genes and expressing the indicated GAL4 derivative (or a vector-alone control) were grown in liquid culture and assayed for β-galactosidase activity. **c**, Primer extension analysis. RNA was isolated from yeast cells bearing the indicated reporter and GAL4 derivative. Constitutive ADH1 transcription from the alcohol dehydrogenase gene was used as an internal control for variations in total cellular RNA. Positions of the GAL1/lacZ and ADH1 transcripts are indicated on the left-hand side of the gel. **d**, Repression of weak activation. Line 1, repression of a weakly activating GCN4 allele. A *gcn4*[−] yeast strain containing an integrated reporter gene

as shown was co-transformed with single-copy plasmids expressing either *gcn4*-C163 (ref. 5) or wild-type GCN4 and an expression vector encoding GAL4(1-147) + SSB24. Line 2, repression of Dorsal-driven activation. The depicted reporter was integrated into a Dorsal expressing strain and into the parent strain, which does not express Dorsal. Cells were transformed with plasmids expressing the indicated GAL4 derivatives. The observed activation by Dorsal depends upon the presence of a Dorsal binding site, and the repression by GAL4(1-147) + SSB24 requires GAL4 binding sites (not shown). Line 3, repression of GCN4 bound further upstream of TATA. A yeast strain containing the depicted reporter was transformed with plasmids expressing the indicated GAL4 derivatives. Cells were grown in liquid culture and assayed for β-galactosidase activity. Standard procedures were used for all strain and plasmid constructions and activity assays (details available on request from the authors).

c

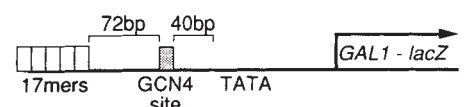
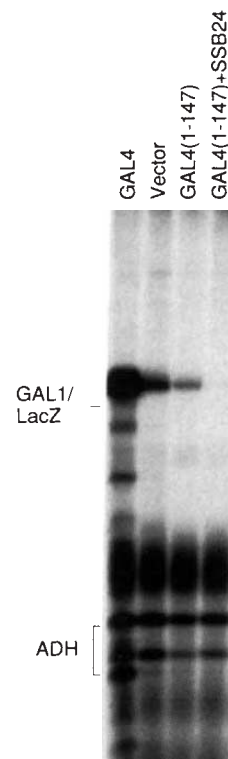
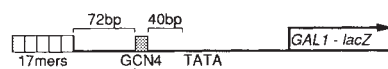


FIG. 2 Specificity of repression by GAL4(1-147) + SSB24 and GAL4(1-147) + SSBM7. *a*, Repression of GCN4-stimulated transcription. Cells containing the indicated reporter and repressor were grown in liquid culture and assayed for β -galactosidase activity. *b*, Repression of activation elicited by the UAS_U . Cells containing the indicated reporter and repressor were grown in liquid culture and assayed for β -galactosidase activity. *c*, Repression of activators from the UAS_U as measured by genetic selection on plates. 10^4 cells containing the depicted reporter and the indicated GAL4 derivative, grown in liquid culture, were spread onto plates containing 5-fluoroorotic acid (5 FOA). Survival on this medium requires repression of the *URA3* gene. As shown, some 30% of the cells bearing GAL4(1-147) + SSBM7 formed colonies on these plates, whereas less than 0.1% of the cells bearing GAL4(1-147) + SSB24 or GAL4(1-147) formed colonies. GAL4(1-147) + SSBM7 was isolated as a mutant of GAL4(1-147) + SSB24, which, by a colony colour assay, repressed transcription more efficiently than did GAL4(1-147) + SSB24 on a promoter driven by isolated BAS1 and BAS2 binding sites²⁰. Subsequent β -galactosidase assays performed on liquid cultures revealed that GAL4(1-147) + SSBM7 repressed this promoter only slightly more efficiently than did GAL4(1-147) + SSB24 (data not shown). We do not know the identity of the activator(s) bound to the UAS_U that responds differentially to the

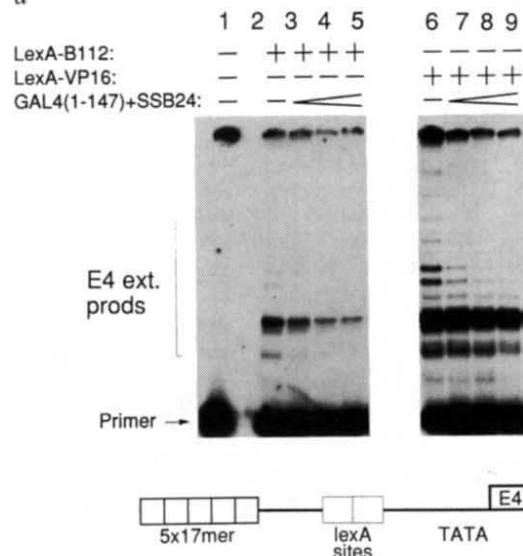
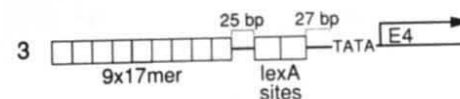
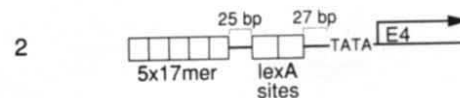
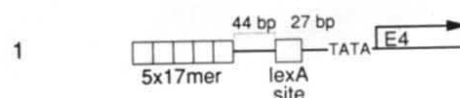
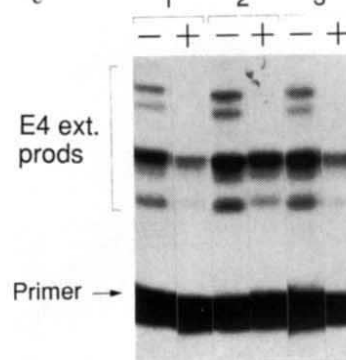
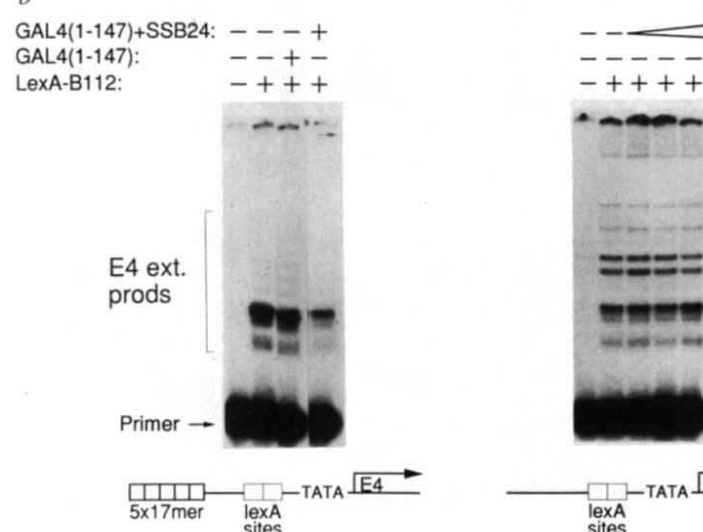
a*b**c*

	β -Galactosidase activity
—	840
GAL4(1-147)	450
GAL4(1-147) + SSB24	60
GAL4(1-147) + SSBM7	120

	β -Galactosidase activity
—	5.0
GAL4(1-147)	4.6
GAL4(1-147) + SSB24	2.0
GAL4(1-147) + SSBM7	<0.1

	FOA ^R Colonies/ 10,000 tested
GAL4(1-147)	1
GAL4(1-147) + SSB24	1
GAL4(1-147) + SSBM7	3,000

two repressors. PPR1, an activator that binds to the UAS_U ¹², responds equally well to the two repressors (not shown). The 2.5-fold repression of the UAS_U promoter observed with GAL4(1-147) + SSB24 could be accounted for by its effect on PPR1, because deletion of the PPR1 binding site results in a similar decrease in activation (3-fold)¹². Evidently some other activator bound to the UAS_U responds to GAL4(1-147) + SSBM7, but not to GAL4(1-147) + SSB24.

a*c**b*

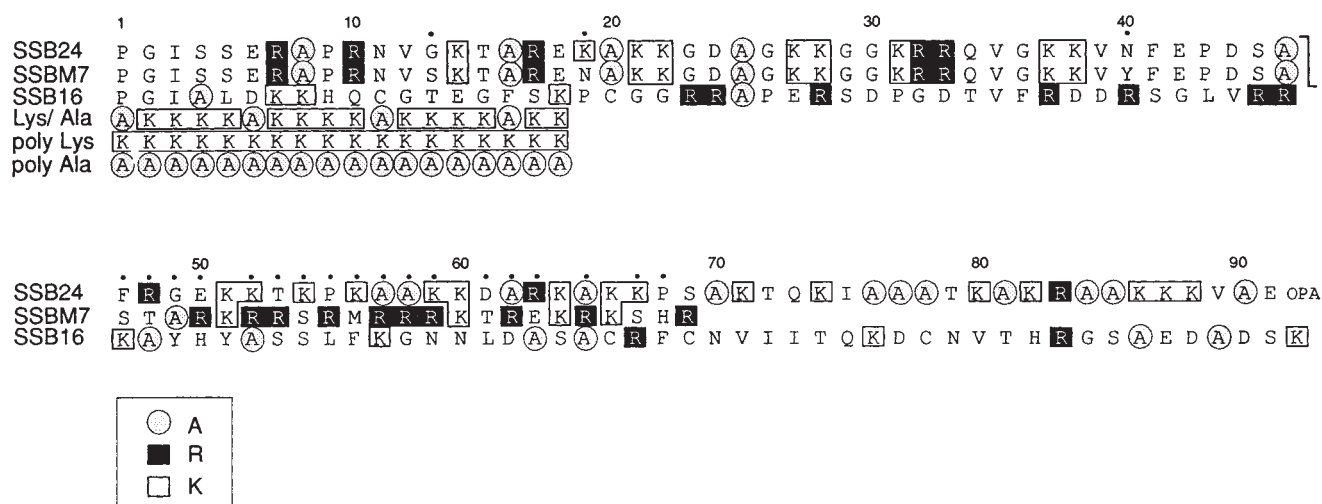


FIG. 4 Protein sequences of SSB24, SSBM7 and SSB16. Dots indicate residues that differ at corresponding positions of the SSB24 and SSBM7 sequences. The bracket indicates the position of the frameshift in SSB24 that generates the carboxy portion of M7. Also included are the sequences of the three peptides; polylysine, Lys/Ala and polyalanine. SSB24 is very similar in sequence to the lysine-rich carboxy-terminal region of histones

H1 and H5 (ref. 24). There is an even stronger match with H1/H5 when the SSB24 protein sequence is examined backwards, however, indicating that this similarity is probably a spurious consequence of the highly skewed (lysine-rich) composition of these molecules. Database search was done at the NCBI using the BLAST network service.

FIG. 3 Repression from upstream *in vitro*. *a*, Repression of a weak and a strong activator. The indicated template was transcribed in yeast nuclear extracts with no activator (lane 1), a constant amount of the weak activator LexA-B112 (lanes 2–5), or a constant amount of the strong activator LexA-VP16 (lanes 6–9). Increasing amounts of GAL4(1–147) + SSB24 were added as indicated. The templates used in these experiments were derivatives of the adenovirus E4 promoter containing the E4 TATA box and 260 bp of E4 coding sequence²¹. Each template also contained one or two consensus LexA binding sites 27 bp upstream of TATA and, in some cases, GAL4 binding sites further upstream. The start sites of the two major transcripts, approximately 50 bp downstream of TATA, are typical of yeast genes transcribed in yeast cells and cell-free extracts²². One of several minor transcripts begins at the position of the major start site generated from the E4 gene in HeLa cell extracts (~20 bp downstream of TATA). As shown here, GAL4(1–147) + SSB24 efficiently repressed both major and minor transcripts stimulated by LexA-B112, as well as the minor transcripts generated by LexA-VP16. But repression of the major transcripts, elicited by LexA-VP16, was barely discernible. Activation of the E4 promoter depended on the presence of activator binding sites (data not shown). Under the conditions used here, basal level transcription was barely detectable (lane 1), and activation from these templates was readily observed (8–12 fold for B112 and 20–30 fold for VP16). *b*, Requirement for the SSB24 repressing region and the presence of GAL4 binding sites in the template. The indicated templates were transcribed as in *a* without activator (lanes 1 and 5), a constant amount of LexA-B112 (lanes 2–4 and 6–9). GAL4(1–147) (lane 2), or GAL4(1–147) + SSB24 (lane 4). Lanes 7–9 contain the same titration of GAL4(1–147) + SSB24 as in *a*. *c*, The effect of changing the number of repressor and activator binding sites. The indicated templates were transcribed as in *a* in the presence of LexA-VP16 with (+) or without (–) GAL4(1–147) + SSB24. Transcription products were analysed by primer extension. E4 primer extension products are indicated on the left. Transcription reactions were as described²³, with modifications: the concentration of DNA template was 20 pM and each reaction contained 12 mg ml⁻¹ pGEM3ZF (Promega) plasmid DNA as carrier. Increasing concentrations of GAL4(1–147) + SSB24 (from 5.7–51 nM in threefold steps) or, in *b*, GAL4(1–147) (27.4 nM) (lane 2) or GAL4(1–147) + SSB24 (17 nM) (lane 3), were preincubated with template DNA and yeast nuclear extracts (80 µg) for 5 min at 21 °C. Either LexA + B112 (15 nM) or LexA + VP16 (25 nM) were added to the reactions and they were incubated at 21 °C for 5 min. Nucleotides were added and transcription allowed to proceed at 21 °C for 40 min. In *c*, GAL4(1–147) + SSB24 concentration was 51 nM and the carrier was 10 mg ml⁻¹. Protein purification, extract preparation and plasmid construction followed standard procedures (details available on request from the authors). LexA-VP16 protein purification will be published elsewhere (M. Su, personal communication).

of +25 and +21, respectively, whereas the weakly repressing region SSB16 bears a net positive charge of +4. SSBM7 is particularly rich in arginine residues, whereas SSB24 is rich in lysine and alanine residues. The peptide designated Lys/Ala was designed to mimic the salient features of SSB24 and, as shown in Fig. 1b (line 1), this peptide repressed almost as efficiently as SSB24 when tethered upstream of a single GCN4 site. The peptide Lys/Ala is a more efficient repressing region than either polylysine or polyalanine (see Fig. 1b and 4), and GAL4(1–147) fused to any of these peptides had no effect in the absence of GAL4 binding sites. The peptide Lys/Ala mimicked SSB24 in another regard: when tethered upstream of the *URA3* promoter it failed to repress (data not shown).

Our data are plausibly explained by assuming that our new highly basic repressing regions work by interacting with activators bound nearby and preventing the latter from stimulating transcription, although we cannot exclude the possibility that our repressing regions interact with some component(s) of the basic transcriptional machinery (refs 14, 15, and B. M. Herschbach and A. D. Johnson, in the press). The demonstration of repression *in vitro* argues against any model in which our repressors would work by sequestering DNA templates in cellular compartments inaccessible to RNA polymerase. Moreover, our extracts were supplemented neither with histones nor with nucleosome organizers¹⁶, and so it is unlikely that nucleosome formation is involved. The finding that our Lys/Ala peptide works as a more efficient repressing region than polylysine suggests that the positive charge found in the Lys/Ala peptide and in our new repressing regions is necessary but not sufficient. The *Drosophila* proteins Eve and Krüppel, as well as the yeast proteins $\alpha 2$ /mcm1 and LexA-SSN6, have been reported to repress when bound upstream of a target gene. A basic region of Eve has been implicated in the repressing function¹⁴, and for Krüppel the identity of the repressing regions is controversial^{17,18}. $\alpha 2$ /mcm1 probably works (unlike GAL4(1–147) + SSB24) by recruiting SSN6 and TUP-1 (ref. 6), and repressing regions on these proteins have not been identified. We therefore do not know whether positive charge, in the appropriate sequence context, will be a common attribute of natural repressors.

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1. Ma, J. & Ptashne, M. *Cell* **51**, 113–119 (1987).
2. Ptashne, M. *A Genetic Switch: Phage Lambda and Higher Organisms* (Cell Press and Blackwell Scientific, Cambridge, MA, 1992).
3. Ptashne, M. & Gann, A. A. F. *Nature* **346**, 329–331 (1990).
4. Lewin, B. *Cell* **61**, 1161–1164 (1990).
5. Hope, I. A. & Struhl, K. *Cell* **46**, 885–894 (1986).
6. Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M. & Johnson, A. D. *Cell* **68**, 709–719 (1992).
7. Schultz, J. & Carlson, M. *Molec. cell. Biol.* **7**, 3637–3645 (1987).
8. Steward, R. *Science* **238**, 692–694 (1987).
9. Kamens, J. & Brent, R. *New Biol.* **3**, 1005–1013 (1991).
10. Ray, R. P., Arora, K., Nüsslein-Volhard, C. & Gelbart, W. M. *Development* **113**, 35–54 (1991).
11. Jiang, J., Kosman, D., Ip, Y. T. & Levine, M. *Genes Dev.* **5**, 1881–1891 (1991).
12. Roy, A., Exinger, F. & Losson, R. *Molec. cell. Biol.* **10**, 5257–5270 (1990).
13. Ruden, D. M., Ma, J., Li, Y., Wood, K. & Ptashne, M. *Nature* **350**, 250–252 (1991).
14. Han, K. & Manley, J. L. *Genes Dev.* **7**, 491–503 (1993).
15. Johnson, F. B. & Krasnow, M. A. *Genes Dev.* **6**, 2177–2189 (1992).
16. Workman, J. L. & Roeder, R. G. *Cell* **51**, 613–622 (1987).

17. Licht, J. D., Gossel, M. J., Figge, J. & Hansen, U. M. *Nature* **346**, 76–79 (1990).
18. Sauer, F. & Jäckle, H. *Nature* **353**, 563–566 (1991).
19. Ma, J. & Ptashne, M. *Cell* **48**, 847–853 (1987).
20. Devlin, C., Kimberly, T.-B., Shore, D. & Arndt, K. T. *Molec. cell. Biol.* **11**, 3642–3651 (1991).
21. Lin, Y.-S., Carey, M. F., Ptashne, M. & Green, M. R. *Cell* **54**, 659–664 (1988).
22. Lue, N. F., Flanagan, P. M., Sugimoto, K. & Kornberg, R. D. *Science* **246**, 661–664 (1989).
23. Ponticelli, A. S. & Struhl, K. *Molec. cell. Biol.* **10**, 2832–2839 (1990).
24. van Holde, K. E. *Chromatin* (Springer, New York, 1989).

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Protein/nucleotide sequences should ideally be in the three-letter and not the single-letter code for amino acids. One column width of *Nature* can accommodate 20 amino acids or 60 base pairs.

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Immunological toolkits

New products in the field of immunology include an interleukin-1 drug screening assay system, microplate washers and readers, radiolabelled interleukin-4, and a selection of monoclonal and polyclonal antibodies.

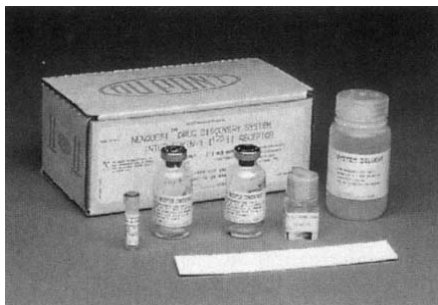
BECTON Dickinson has announced the availability of Results Assurance — a complete family of **quality control products and services available for flow cytometry** (*Reader Service No. 100*). To help flow cytometry laboratories meet their quality control objectives (as mandated in the United States by CLIA '88 regulations and by NCCLS and CDC guidelines), Becton Dickinson is providing the tools to achieve the optimal level of system performance with products and procedures for instrument validation, instrument monitoring, method controls and data quality control. Under the heading of instrument validation and monitoring tools, the company is offering a combination of CaliBRITE beads and AutoCOMP software that automatically provides proper set-up and verification of FACScan



Becton Dickinson's line of quality control products and services for flow cytometry.

flow cytometers, QC log sheets that provide an easy-to-use format to log and track AutoCOMP values for instrument verification and to record set-up and maintenance procedures, and a DNA quality control particles kit. Tools for method control include a positive control for immunophenotyping, a control for reticulocyte enumeration, negative controls, a gating reagent, and data quality control checks.

Du Pont's new **interleukin-1 drug screening system** uses the cloned interleukin-1 (Type I) receptor to provide the target and selectivity needed for the screening of drugs (*Reader Service No. 101*). The high affinity NENQUEST assay both identifies and determines the relative potency of compounds that compete with the binding of [¹²⁵I]-Interleukin-1 to its receptors. It is suitable for targeted high-throughput screening within five hours, as well as the characterization of lead compounds. Each system contains a ready-to-use, stable, cloned human receptor preparation expressed in Chinese hamster ovary cells, a high specific activity [¹²⁵I]-Interleukin-1 tracer, a reference compound and optimized protocol. Systems are supplied in 200- or 1,000-tube formats. The



Du Pont's IL-1 drug screening system.

receptor preparation is available separately. IL-1 is a multifunctional cytokine that plays a central role in inflammation, tissue remodelling, T-cell response and haematopoiesis.

The Midgard **precision current source** is designed for transporting charged molecules, such as proteins and neurotransmitter markers, out of a fine-tip glass micropipette into tissue without any net volume outflow (*Reader Service No. 102*). The Midgard, now manufactured by Stoelting, can drive its maximum d.c. current of 20 μ A through glass micropipettes with impedances of up to 160 M Ω . The level of the current is feedback regulated with a compliance voltage of 2,000 V. A ten-turn pot allows precise settings within the 20- μ A range. On-off cycling can be set on the current source, or gated by an external digital signal.

AMAC announces the availability of OptiClone monoclonal antibodies and OptiLyse lysing solution for use in flow cytometry (*Reader Service No. 103*). OptiLyse **lysing solution** is an erythrolytic reagent intended for lysing human red blood cells, following direct immunofluorescent



Optimized flow cytometry reagents — no-wash lyse and pre-titrated antibodies.

staining of human peripheral white blood cells with OptiClone monoclonal antibodies in preparation for analysis by flow cytometry. OptiLyse lysing solution performs lysing and fixing without instrumentation and is available in bottles with sufficient reagent for 250 tests (for research use only). OptiClone monoclonal antibodies are already optimized and pre-titrated to perform

with 'no-wash' lyse. CD3 FITC/CD4 PE, CD3 FITC/CD8 PE, CD3 FITC/CD19 PE, CD4 FITC/CD8 PE, CD8 FITC/CD4 PE, CD45 FITC/CD14 PE, CD3 FITC/CD16 PE, IgG1 FITC/IgG1 PE and IgG1 FITC/IgG2a PE dual-colour antibodies are available in vials with sufficient reagents for 50 tests. These products are unapproved products being temporarily supplied for essential health purposes.

■ Assorted reagents

Polysciences has formulated a new **tetrazolium compound** for use in nonradioactive cell growth assays and cytotoxicity assays (*Reader Service No. 104*). The compound, MTS, reduces to a water-soluble formazan, which eliminates the need for solubilization steps using volatile organic solvents. In contrast to other water-soluble tetrazoliums (XTT) which must be used promptly after dilution, Polysciences says that MTS solutions are much more stable. MTS solutions can also be safely frozen for long-term storage. Data sheets are available for the full line of tetrazolium salt redox indicators.

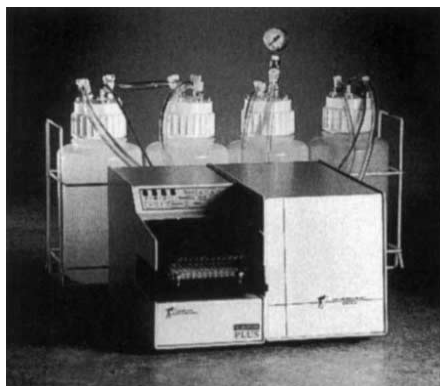
Stratech Scientific is distributing **heparan sulphate proteoglycan**, which has been manufactured by Collaborative Biomedical (*Reader Service No. 105*). Heparan sulphate proteoglycan has been found in the basement membrane of a variety of tissues including skin, liver, blood vessels, kidneys and basement membrane-rich tumour, Engelbreth-Holm-Swam (EHS) mouse sarcoma. It is composed of polysaccharide chains that are covalently linked to a core protein and has been described as a multidomained and multifunctional molecule that binds growth factors and extracellular matrix molecules such as type-IV collagen, laminin and fibronectin. Such interactions may result in the control of cell proliferation and differentiation. Heparan sulphate proteoglycan isolated from the EHS tumour is also said to stimulate DNA synthesis in fetal bovine heart cells.

Kretech says that with the aid of a newly developed **target unmasking fluid**, immunohistochemistry on (archival) paraffin embedded tissues and frozen sections is now possible with a large number of formerly less successful monoclonal and polyclonal antibodies (*Reader Service No. 106*). Staining intensity and frequency are improved by a factor of at least ten, the company says. Target unmasking fluid does not contain heavy metals or carcinogenic compounds. Sections have to be incubated in target unmasking fluid for only 10 min at 90 °C using

a waterbath or microwave oven according to the normal routine immunohistochemistry protocol. The reagent has been used successfully for p53, PCNA, S-100, MIB-1, collagen 1, 3 and 6, CD44, HLA DR, keratins and P-glycoprotein.

■ Microplate instrumentation

Dynatech Laboratories says that flexibility is the key to its newly introduced Ultrawash Plus **microplate washer**, which features switchable heads that can be manually switched between 96-well and row wash formats or 96-well and columnar formats (*Reader Service No. 107*). In addition to offering switchable wash configurations, the



Ultrawash Plus with a switchable wash configuration.

Ultrawash Plus can store up to 20 user-defined wash protocols as well as five different microplate settings. The system can cope with U, V, C and flat-bottomed wells. An automatic clean cycle flushes the entire system, and the dispense empty and waste full indicators are designed to prevent the user from having to abort and reinitiate an assay. The system is supplied with the washer unit, vacuum/pressure unit, non-corrosive quick disconnect tubing and a lightweight, transportable bottle-rack assembly.

Bio-Tek has introduced an **automated microplate washer and a scanning microplate reader** (*Reader Service No. 108*). The EL403H microplate washer is designed to wash 96-well microplates in only 8 s. The five stored wash programs control wash



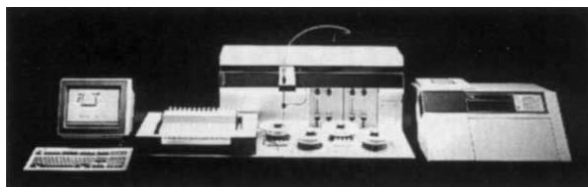
Automated plate reader from Bio-Tek.

parameters of volume, cycles, soak time, height adjustments and residual removal. It is suitable not only for standard ELISAs, FIAs and LIAs, but also for those assays where cellular layers on the bottom of the microplate need to be washed and protected from unnecessary disturbance. Bio-Tek says that it should be useful for HIV and hepatitis ELISAs. Building on the features of the EL309 series, the EL311 scanning microplate reader interfaces directly with a CRT for instant display of absorbance data and reports. The 'Scan-A-Well' feature provides up to three data points across each well for agglutination for MIC-type analysis. Rapid, repeated reading of desired rows or columns of wells is provided for kinetics applications. Computer control is standard; an internal universal barcode reader is optional. The instrument can be combined with the company's new Turbo Accelerator, a compact data analysis unit.

■ Immunoassays

Malakit M2 is the new anti-mitochondrial M2 ELISA kit from Biolab that is designed for the **detection of M2-type anti-mitochondrial antibodies**, which are considered as important and specific serological markers of primary biliary cirrhosis (*Reader Service No. 109*). Current IFA techniques can be time-consuming and frequently lead to confusion with other types of anti-mitochondrial antibodies. Malakit M2, allowing determination of both IgG and IgM anti-M2 antibodies, uses a purified antigenic complex including the different determinants of pyruvate dehydrogenase that are highly immunogenic and very specific.

A new modular **information management system** designed to reduce manual involvement in immunoassay research has been developed jointly by Tecan AG and Abbott (*Reader Service No. 110*). The Lintax plus system supports information management, retrieval and archiving functions. The system was developed to link the Abbott TDx or IMx clinical immunoassay analysers with Tecan's instruments via common hardware and data management software. The result, the companies say, is an integrated assay system, with sample and control preparation being carried out by a Tecan robotic sample processor, followed by transfer to an Abbott carousel before analysis. In terms of hardware, the Lintax plus consists of a PC and data concentrator together with a Tecan robotic sample processor and one or more TDx or IMx analysers. The PC, with printer and barcode wand reader, is used for the entry of sample identifications and test requests. These data can be input directly at the PC or downloaded from a host computer. After dispensing of samples by the robotic sample processor and analysis on the TDx/IMx, the results are collected via the DacoBox



Lintax plus modular information management system.

data concentrator and transferred to the Lintax plus PC. They can then be analysed, formatted and output or archived. At the heart of the Lintax plus system is the software, and particularly the data management features. This handles all the information management functions. There is scope for defining tests including units of measurement and cut-off limits, customization of the contents and format of report listings, and various operational modes for the system including password protection and the running of external programmes embedded within Lintax plus.

The sELAM-1 ELISA from Bender MedSystems is an enzyme-linked immunosorbent assay for the **quantitative detection of soluble endothelial leukocyte adhesion molecule-1 (sELAM-1)** levels in cell culture supernatants, human serum, plasma, amniotic or other body fluids (*Reader Service No. 111*). The assay is designed to be rapid, requiring only a 2 h 15 min incubation, and has a stated sensitivity of less than 1.6 ng ml^{-1} ($1.6\text{--}50 \text{ ng ml}^{-1}$ dynamic range). ELAM-1, also known as E-selectin, vascular selectin and LECAM-2, is a 115-kDa single-chain glycoprotein and belongs to the selectin family of adhesion molecules. ELAM-1 expression is restricted to cytokine activated endothelial cells and mediates their initial interaction with neutrophils in areas of inflammation. Levels of sELAM-1 have been demonstrated in normal human sera and the monitoring of sELAM-1 appears to be of prognostic value in several pathological conditions such as allergy, cancer, infectious diseases, rheumatology and transplant rejection.

■ Interleukins

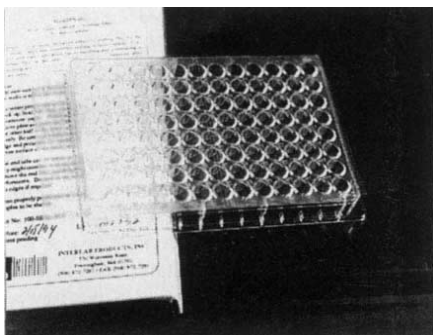
Interleukin-4 (IL-4), an essential immune-system mediator, is now available with a [^{125}I] label in a well-characterized pure state from Amersham (*Reader Service No. 112*). This **radiolabelled lymphokine** is purified by reverse-phase HPLC to give a product of high radiochemical purity and reliable specific radioactivity. Also known as B-cell growth factor, T-cell growth factor and mast cell growth factor, IL-4 has been shown to be an important mediator within the immune system. As such, radiolabelled IL-4 will be useful in studies of allergic diseases using radioimmunoassays and receptor binding assays, amongst other techniques.

Recombinant human interleukin-10 (IL-10) is now available from Genzyme (*Reader Service No. 113*). This cytokine is

produced by the Th2 helper cells, B cell subsets and LPS-activated human monocytes. IL-10 was first characterized as an immune system suppressor based on its ability to inhibit Th1 helper cell synthesis of cytokines and also macrophage/monocyte production of inflammatory cytokines. IL-10 has also been shown to have stimulatory capabilities such as inducing proliferation and differentiation of B cells into antibody secreting cells and also promoting *in vitro* growth of IL-2/IL-4-activated mouse thymocytes. Genzyme's rIL-10 is active in both mouse and human systems.

Handy helpers

InterLab Products has developed SealPlate adhesive film for the sealing of microplates (Reader Service No. 114). The adhesive film has perforated end tabs allowing easier, more accurate positioning of the film over the plates and, therefore, more secure seals, InterLab says. SealPlate is inert, both the



SealPlate microplate adhesive film.

polyester film as well as the Acroolefin adhesive, making SealPlate compatible with virtually all procedures performed in microplates. SealPlate is packaged 100 per box and can be used between temperatures of 30 °C and 110 °C. It is available both sterile and nonsterile. The acrylic base of the adhesive will resist most petroleum solvents and lower alcohols.

The Fro-Tissuier — for frozen sections — and the Para-Tissuier — for paraffin sections — pens available from the Binding Site are designed to prevent tissue sections from falling off, moving or wrinkling on the microscope (Reader Service No. 115). Each pen provides a sticky membrane, which is applied to the slide and onto which the frozen or paraffin section is placed. Multiple sections, separated by individual membranes, can be applied to the same slide and can easily be compared. The pens are intended for use in immunostaining methods, specific procedures requiring high temperatures and rigorous techniques such as *in situ* hybridization.

Monoclonals

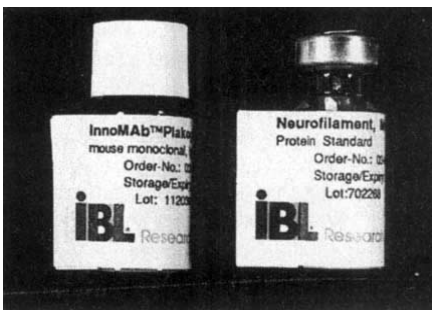
New from Advanced Biotechnologies — a polyclonal antibody to gastrin raised in rabbits (Reader Service No. 116). The antibody reacts equally with both gastrin I and II

and recognises sulphated and non-sulphated forms. Advanced Biotechnologies says that the antibody has a one per cent crossreactivity with CCK-8 and CCK-33 and less than 0.1 per cent with pentagastrin, little gastrin 13-17 and little gastrin 8-17. (Assessment is by radioimmunoassay where the assay sensitivity is 0.8 fmol per tube.) Gastrin is a polypeptide of 17 amino acids, secreted by the G-cells of the gastric antral mucosa and by the upper small intestine. The main clinical use of the assay is in the investigation of the Zollinger-Ellison syndrome.

A new polyclonal antibody from Biogenesis could be useful in the investigation of synaptic vesicle proteins (Reader Service No. 117). The antibody, raised in rabbits against purified human synapsin I (a + b) is suitable for use in ELISAs and blotting procedures. Of particular interest for research purposes is its crossreactivity with synapsin from rat brain synaptosomes.

Affinity BioReagents is expanding its line of antibodies to intracellular receptors and heat shock proteins (Reader Service No. 118). New antibodies to retinoic acid receptor alpha and beta, vitamin D receptor and various forms of thyroid hormone receptor are now available to the research community. These research tools add to ABR's line of steroid receptor antibodies, which includes antibodies to androgen, oestrogen, progesterone, glucocorticoid and mineralocorticoid receptors. New polyclonals to HSP84, HSP86 and HSP104 can also be found at ABR. These products complement the company's two monoclonals to HSP90, and five monoclonals to forms of HSP70. In addition, ABR will soon be expanding its anti-HSP product line to include polyclonals to GRP78 and HSP56/FKBP59.

IBL Research Products announces the availability of surface and cytoskeletal monoclonal antibodies and protein standards (InnoMab) (Reader Service No. 119). These surface and cytoskeletal monoclonal antibodies and protein standards include, among others, cytokeratins ranging from #7, 8, 13, 17-20, vimentin, plakoglobin, GFAP, neurofilaments, synaptophysin, desmin, filamin and vinculin. These products should be of interest to researchers in the fields of biochemistry, cell biology, dermatology,



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PRODUCT REVIEW

immunology, immunopathology, neuroscience and oncology.

A range of **monoclonal and polyclonal antibodies to extracellular signal-regulated protein kinases (ERKs)** 1, 2 and 3 and to the MAP kinase kinase (MEK) developed by scientists at Transduction Laboratories are now available from Affiniti Research Products (*Reader Service No. 120*). The MAP kinases, also known as ERKs are involved in many signal transduction processes and appear to be central to an evolutionary conserved protein kinase cascade. All the antibodies are suitable for use in western blotting and other immunochemical applications at dilutions in excess of 1:1,000. One of the monoclonal antibodies (clone MK12), which principally recognises ERK-1 is also available in derivatized form, directly conjugated to biotin or horseradish peroxidase, or immobilized on agarose.

New **monoclonal antibodies against integrins** $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$, specific for alpha chains (all of which have been demonstrated to have the ability to block the ligand binding site if attached to the receptor),

are now available from Dako (*Reader Service No. 121*). Characterized by their function of integrating the cytoskeleton of different cells, integrins are heterodimeric cell transmembrane glycoproteins consisting of a noncovalently linked alpha and beta chain. The functional importance of adhesion proteins has been linked to a variety of critical cellular roles. These include the binding of cells to the extracellular matrix and other cells, anchorage-dependent cell growth, differentiation, proliferation, cell migration and homing, as well as the control of polarity and cell shape. In another area, Dako is offering a new polyclonal anti-tau for use on routinely processed tissue. When used in conjunction with Dako anti-beta-amyloid and anti-ubiquitin, this polyclonal antibody may distinguish neurofibrillary tangles from senile plaques. All three of these antibodies may be used on routinely processed, formalin-fixed, paraffin-embedded tissue sections.

Serotec has produced a purified form of the **monoclonal antibody YL 1/2, which recognises the Glu-Glu-Phe (OH) C-terminal epitope of a tubulin** (*Reader Service No. 122*). This antibody has been used in a novel immunoaffinity purification technique that allows rapid purification of HIV reverse transcriptase, GTP-ase, RNase H, and other proteins by adding the Glu-Glu-Phe sequence of the C-terminal of proteins whose function is not compromised by the C-terminal modification so that rapid purification is facilitated.

■ Catalogues

The 1993 Chemicon catalogue lists **immunological reagents** including those used for neuroscience, infectious disease, adhesion molecule/extracellular matrix and cell structure/transport research (*Reader Service No. 123*). The catalogue features diagnostic test kits, bulk reagents and custom services including the production and purification of antibodies. New reagents for this year include antibodies and antigens for environmental compounds, antibodies to mouse cell surface markers and test kits for a wide variety of autoimmune diseases.

The 1993 catalogue from BioGenex Laboratories is now available listing over 250 ready-to-use and concentrated **antibodies, DNA probe test kits and enhanced biotin-streptavidin detection systems** for use in immunohistochemistry and *in situ* hybridization (*Reader Service No. 124*). This year's catalogue contains photographs showing correct tissue staining, and lists over 40 new monoclonal antibodies, as well as new *in situ* detection kits for kappa/lambda light chains. Among the new antibodies for 1993 are monoclonals to many vascularization and CD markers (CD31, CD34), adhesion molecules (ICAM-1, PECAM), lymphoid and myeloid markers (BLA.36), infectious agents (CMV, *Pneumocystis carinii*), and

prognostic markers (BruU, GST [alpha, mu and pi], and p53 [clones DO7, 1801 and polyclonal CM1]). The new Super Sensitive 500 large-volume detection systems offer the enhanced sensitivity of amplified biotin-streptavidin kits in a more economical format, BioGenex says.

Advanced Magnetix presents a 1993-1994 product **catalogue for life science research** (*Reader Service No. 125*). Over 50 BioMag super-paramagnetic particles are featured

including direct attachment procedures and applications for cell separation. A complete list of pre-conjugated BioMag products is described such as magnetic second antibodies, magnetic binding proteins and magnetic anti-human leukocyte antibodies for both positive and negative selection. An extensive line of immunoassays for cytokines, eicosanoids and cyclic nucleotides is offered with specific performance characteristics for each assay. A wide range of immunoassay reagents is also available including antisera, standards, tracers and conjugates. Also detailed is the newly released Apo-Pak, containing reagents for the measurement of cell death (including apoptosis and necrosis research).

A 1993/1994 product catalogue available from Ancell includes an expanding list of **monoclonal antibodies and immunological reagents** for use in research on human immune system antigen presentation (*Reader Service No. 126*). The products lend themselves to use in flow cytometry analysis, immunohistochemistry, cell surface labelling and immunoprecipitation. Structure-function relationships can be investigated via cross competition, inhibition and activation studies. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

Correction

The following individual should be acknowledged as a coauthor to the article "TBASE: A Computerized Database for Transgenic Animals and Targeted Mutations" that appeared in the product review section of *Nature* (363, 375-376; 27 May 1993): Daniel A. Jacobson, Johns Hopkins University, Baltimore, Maryland, USA.



AMI's 1993-1994 product catalogue.

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